University of Alberta

Development of Ganglioside-based Vaccines for Use in Active Tumor Immunotherapy

by

Sandra Jacques ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Chemistry

Edmonton, Alberta
Fall 2008

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-46338-3
Our file Notre référence
ISBN: 978-0-494-46338-3

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

The overexpresssion of gangliosides in melanoma and several other tumors of neuroectodermal origin makes these molecules attractive targets for the development of tumor immunotherapy. To date, moderate immune response to ganglioside-based conjugates and difficulties associated with ganglioside synthesis and conjugation have hampered the development of a viable cancer vaccine.

This work describes the preparation of ganglioside-based conjugates, their evaluation as cancer vaccines, and the development of a new strategy to enhance their immunogenicity. To efficiently gain access to ganglioside analogues amendable to conjugation, a chemoenzymatic synthesis was developed. A lactoside incorporating a truncated ceramide aglycon functionalized for coupling was synthesized chemically on a gram scale. This truncated lactosyl ceramide avoided the extensive hydrophobicity typical of ceramide-containing compounds allowing for enzymatic elaboration of the glycan chain or production of complex gangliosides centered on this core using metabolically engineered bacteria. The truncated glycolipids obtained by this approach were further modified with fatty acid and sphingosine chains of various lengths to generate gangliosides incorporating different ceramide fragments. These glycolipids were conjugated to a carrier protein (tetanus toxoid) *via* an adipate-linker and the ability of the conjugates to induce an immune response was evaluated in mice.

Mice immunized with these glycoconjugates responded with high titers of antigen specific antibodies of the IgM, but predominantly of the IgG subclass. Further investigation of the antibody binding paratope by solid-phase assay showed recognition of both the carbohydrate epitope and elements of the ceramide moiety. It was also shown that the nature of the lipid chain as well as the site of conjugation have an influence on the immunogenicity of the constructs. Glycolipids linked to the carrier protein *via* the fatty acid portion of the molecule were significantly less immunogenic than those incorporating a long fatty acid chain. It can be speculated that the presence of a long fatty acid chain imparts flexibility to the molecule, rendering it more accessible for presentation to the immune system. A novel strategy for augmenting the immunogenicity of ganglioside conjugates in which the antigens are targeted to dendritic cells was also explored. Using this protocol, good titers of ganglioside specific antibodies were obtained with very low concentrations of immunogen. This strategy could allow the development of low-dose vaccines in the future.

Acknowledgements

I would like to thank my supervisor Dr. David Bundle for his guidance and support during my time at the University of Alberta. I feel that joining his group provided me with great learning opportunities.

The work presented in this thesis would not have been possible without the help of several individuals. I would like to thank Mr. Blake Zheng, Ms. Lisa Pritchard, Ms. Joanna Sadowska, and Gordon Grant for their technical assiatance during the course of my degree. I am also greatful to Angie Morales for her expertise in mass spectrometry. Organic chemistry is not an easy discipline, especially when working with large molecules. I think that my degree would not have been as successful without the help of Dr. Chang-Chun Ling. I would like to thank you Chang-Chun for sharing your knowledge with me and always being available for discussion.

I had the priviledge to work under the guidance of an exceptional mentor, Jamie Rich, during the first part of my degree. I would like to thank you, Jamie for sharing your knowledge of chemistry and friendship. You inspired me to be a better student.

During the course of my degree, I have met several people that have made my stay in Edmonton memorable. I would like to thank Magali Buffet, Vincent Bouvet, Scott McGavin, Corwin Nycholat, Frédéric Carrel, and Hyo-Sun Kim for making the long hours spent in the lab enjoyable.

My stay in Edmonton has been brighten by a wonderful person, Karen Anstead. Thank you, Karen for all the conversations we had, your tremendous generosity and your kindness. You were my family in Edmonton.

J'aimerais également remercier mes parents, Jocelyne et Pierre Jacques pour leur support er leur aide. Malgré la distance qui nous sépare, vous avez été constamment présents pour moi. Les gâteries que vous m'avez envoyées et les conversations au téléphone m'ont beaucoup aidée, tout spécialement durant les moments difficiles. Je vous remercie de tout mon cœur de m'avoir encouragé à poursuivre mes rêves.

I have the priviledge to share my life with a wonderful person, my boyfriend, Matt Clay. Matt, I would like to thank you for your support and your understanding (eventhough sometimes it was difficult). You have been there for me during the good and difficult times, making me laugh when I needed it, talk when I felt down, and making me more confident. You help me to be a better person. I love you with all my heart.

Table of Contents

Page

Chapter 1 1

1.1 Carbohydrates in biological systems: Their structure, biosynthesis and significance 1
  1.1.1 Glycoproteins 1
  1.1.2 Glycolipids 3
  1.1.3 Significance of carbohydrate interactions 4

1.2 Carbohydrate synthesis 6
  1.2.1 Chemical methodologies 6
    1.2.1.1 General considerations 6
    1.2.1.2 Glycosyl donors 7
    1.2.1.3 Formation of the 1,2-*trans*-α and 1,2-*trans*-β linkages 8
    1.2.1.4 Formation of 1,2-*cis*-α linkages 9
    1.2.1.5 Formation of 1,2-cis-β linkages 10
  1.2.2 Biological approaches to the synthesis of oligosaccharides 11
    1.2.2.1 Glycosyltransferases 11
    1.2.2.2 Glycosidases 12
    1.2.2.3 Oligosaccharide synthesis by metabolically engineered bacteria 15

1.3 Carbohydrates in drug discovery and development 15

1.4 Conjugate Vaccines 16

1.5 Tumor Immunotherapy 19
  1.5.1 Tumor antigens 19
  1.5.2 Gangliosides in tumor immunotherapy 22

1.6 Scope of the project 24

Chapter 2 26

2.1 Chemistry of Sphingosine and Gangliosides 26
  2.1.1 Previous synthesis of sphingosine 26
  2.1.2 Previous chemical synthesis of gangliosides 28

2.2 Enzymatic methods for the synthesis of gangliosides 36
  2.2.1 Glycosyltransferases in the synthesis of gangliosides 37
  2.2.2 Glycosidases in the synthesis of gangliosides 38

2.3 Target structures 40

2.4 Results and Discussion 42
  2.4.1 Synthesis of truncated lactosyl sphingosine analogue 11 42
  2.4.2 Chemoenzymatic synthesis of GM_3 and GM_2 ganglioside analogues 45
    2.4.2.1 Preparation of cytosine-5'-monophosphate *N*-acetyl neuraminic acid (CMP-Neu5Ac) 46
    2.4.2.2 Enzymatic sialylation to form a GM_3 analogue 47
    2.4.2.3 Enzymatic preparation of a GM_2 analogue 48
  2.4.3 Derivation of ganglioside analogues with different acyl chains 50
  2.4.4 Alternative Route to Gangliosides 51

2.5 Conclusions 53

Chapter 3 **54**

3.1 Oligosaccharide synthesis by metabolically engineered bacteria 54

3.2 The 'living factory' approach: Factors to be considered 56
  3.2.1 Host strains 57
  3.2.2 Sugar nucleotide donors 57
  3.2.3 Acceptors 58

3.3 Results and discussion 59
  3.3.1 Concepts and methodology relating to the production of gangliosides in *E. coli* 59

3.3.2 Preparation of GM$_3$ Analogue 24 65
3.3.3 Production of GD$_3$ Analogue 25 67
3.3.4 Production of GM$_2$ and GD$_2$ Derivatives 26 and 27 70
3.3.5 Production of GM$_1$ analogue 28 74
3.3.6 Preparation of GT$_{1a}$ Derivative 29 77

3.4 Conclusions 79

Chapter 4 **81**

4.1 Tumor immunotherapy: significance of gangliosides 81

4.2 Humoral immune response: Factors to consider 82

4.3 Immunization: Experimental considerations 83
4.3.1 Carrier protein 84
4.3.2 Conjugation strategy 85
4.3.3 Immunization protocol 86

4.4 Results and discussion 87
4.4.1 Synthesis of ganglioside conjugates and inhibitors for immunization study 87
4.4.1.1 Preparation of ganglioside conjugates 87
4.4.1.2 Synthesis of soluble ganglioside inhibitors 91
4.4.2 In vivo evaluation of the immune response generated by ganglioside-tetanus toxoid conjugates 92
4.4.2.1 Immunization 92
4.4.2.2 Determination of antibody titer for mice immunized with GM$_3$-TT conjugate 92
4.4.2.3 Analysis of immune sera obtained from mice immunized with GM$_3$-TT conjugate 34 by competitive ELISA 96
4.4.2.4 Determination of antibody titer for mice immunized with GM$_2$-TT conjugate 35 98

4.4.2.5 Analysis of immune sera obtained from mice immunized with GM_2-TT conjugate 35 by competitive ELISA 99
4.4.3 Analysis of the effect of the aglycon on ganglioside immunogenicity 101
4.4.3.1 Preparation of ganglioside conjugates incorporating different lipid fragments 101
4.4.3.2 Determination of antibody titers for mice immunized with ganglioside conjugates incorporating different lipid fragments 104
4.4.3.3 Evaluation of antibody binding on melanoma cells 106

4.5 Conclusions 107

Chapter 5 **109**

5.1 Role of dendritic cells in the immune system 109

5.2 Dendritic cells and cancer immunotherapy 110

5.3 Enhancing the immune response to gangliosides using a bispecific monoclonal antibody 111

5.4 Results and discussion 113
5.4.1 Development of a bispecific fusion protein 113
5.4.2 Biotinylation of ganglioside antigens 116
5.4.2.1 Synthesis of biotinylated GM_3- and GM_2- glycolipids 117
5.4.3 In vivo evaluation of the immune response generated by the concomitant injection of biotinylated gangliosides and the bispecific fusion protein 118
5.4.4 Preparation of a biotinylated glycopeptide 122
5.4.5 In vivo evaluation of the immune response generated by the concomitant injection of a biotinylated glycopeptide and the P125 bispecific monoclonal antibody 128

5.5 Conclusions 129

Chapter 6 **130**

Chapter 7 133

References 190

Appendix 1 205

List of Tables

Table	Title	Page
Table 3.1	Gangliosides produced using the 'living factory' approach.	65
Table 5.1	Immunization protocol for evaluating the humoral and cell-mediated immune response to biotinylated ganglioside antigens **64** and **65** in mice. (n=5 for each group).	119
Table 5.2	Immunization protocol for evaluating the humoral and cell-mediated immune response to biotinylated glycopeptide antigen **66** in mice. (n=5 for each group).	128

List of Figures

Figure Title Page

Figure 1.1 Core structure of *N*-linked glycoproteins. 2

Figure 1.2 Core structure of *O*-linked glycoproteins. 3

Figure 1.3 Basic structure of (a) glycosphingolipids and (b) glycophospholipids. 5

Figure 1.4 General mechanism of glycosylation reactions. 7

Figure 1.5 Types of glycosidic linkages. 8

Figure 1.6 Neighboring group participation in the formation of 1,2-trans glycosidic linkages. 8

Figure 1.7 Formation of 1,2-cis-α linkages. 10

Figure 1.8 Mechanism of (a) retaining and (b) inverting glycosyltransferases. 13

Figure 1.9 Mechanism of (a) retaining and (b) inverting glycosidases. 14

Figure 1.10 Mechanism of glycosynthases. 15

Figure 1.11 Humoral immune response to carbohydrate antigens. 18

Figure 1.12 Examples of tumor associated carbohydrate antigens. Glycolipids are depicted in part (a) and glycoproteins in part (b). 21

Figure 2.1 Structural features of glycosphingolipids. 27

Figure 2.2 Strategies for the synthesis of sphingosine 1a Olefin cross-metathesis between a protected truncated sphingosine derived from glucose and 1-pentadecene. 1b Wittig coupling of an aldehyde and tetradecyltriphenylphosphonium bromide. 1c. A protected derivative of D-tartrate is treated under the Kocienski modification of the Julia olefination protocol. 2a. Sharpless asymmetric epoxidation is used to construct a truncated aldehyde derivative which can be further extended using the Wittig coupling or Julia olefination. 2b. Asymmetric aldol condensation yield a shorten sphingosine derivative which can be further elaborated to the full lipid. 28

Figure 2.3 Structure of common sialic acids. 29

Figure 2.4 Common sialosyl linkages. 30

Figure 2.5 Problems associated with chemical sialylation. (a) Formation of sialosyl glycal via 2,3-elimination. (b) Example of the use of neighboring group participation in directing the stereochemistry of a glycosylation reaction. 31

Figure 2.6 Direct chemical methods for the formation of sialosyl linkages. 32

Figure 2.7 Directing α-product using solvent participation in sialylation reactions. 33

Figure 2.8 Directing α-sialoside selectivity using auxiliaries. (a) Long-range participation of a C-1 auxiliary. (b) Neighboring participation of a C-3 auxiliary. 35

Figure 2.9 Strategies for the synthesis of glycosphingolipids. 36

Figure 2.10 Interactions (a) disfavoring / (b) favoring glycosylations with sphingosine derivatives. 36

Figure 2.11 Enzymatic sialylation coupled with sugar nucleotide donor regeneration. 38

Figure 2.12 Glycosynthase-mediated synthesis of glycosphingolipids.(a) Synthesis of GM_3 using *N*-palmitoyl-D-erythro-sphingosine as an acceptor. (b) Synthesis of GM_3 using D-erythro-sphingosine as an acceptor followed by subsequent acylation with *N*- palmitoyloxysuccinimide. 39

Figure 2.13 Target Structures. 41

Figure 3.1 (a) Oligosaccharide synthesis by the 'bacterial coupling' approach. (b) Oligosaccharide synthesis by the 'living factory' approach. 56

Figure 3.2 Targeted ganglioside analogues. 60

Figure 3.3 High cell-density culture strategy.[1] 64

Figure 3.4 Biosynthesis of GM_3 analogue **24** by the strain DC7. 66

Figure 3.5 TLC plate analysis of the oligosaccharide content in intracellular (lanes 4-7) and extracellular (lanes 11-14) fractions of samples withdrawn from the culture of GM_3 producing DC7 strain. Standards are in lanes 1 and 8 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 9 (GM_3 24) and lanes 3 and 10 (GD_3 25). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent. 67

Figure 3.6 Biosynthesis of GM_3 **24** and GD_3 **25** by strain NF20. 69

Figure 3.7 TLC plate analysis of the oligosaccharide content in intracellular (lanes 3-5) and extracellular (lanes 8-10) fractions of samples withdrawn from the culture of GM_3 and GD_3 producing NF20 strain. Standards are in lanes 1 and 6 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 7 (lactose and lactosyl sphingosine 11). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent. A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent. 70

Figure 3.8 Biosynthesis of GM_2 and GD_2 analogues **26** and **27** by strain ZWU12. 72

Figure 3.9 Possible by-product formation in the synthesis of GM_2 26 and GD_2 **27** analogues. 73

Figure 3.10 TLC plate analysis of the oligosaccharide content in intracellular (lanes 3-6) and extracellular (lanes 9-12) fractions of samples withdrawn from the culture of GM_2 and GD_2 producing ZWU12 strain. Standards are in lanes 1 and 7 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 8 (lactosyl sphingosine 11). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent. 74

Figure 3.11 Biosynthesis of GM_1 **28** by strain DC23. 75

Figure 3.12 Possible by-product formation in the synthesis of GM_1 analogue **28**. 76

Figure 3.13 TLC plate analysis of the oligosaccharide content in intracellular (lanes 10-14) and extracellular (lanes 3-7) fractions of samples withdrawn from the culture of GM_1 producing DC23 strain. Standards are in lanes 1 and 8 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 9 (lactosyl sphingosine 11). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent. 77

Figure 3.14 Biosynthesis of GT_{1a} derivative **29** by strain NF17. 78

Figure 3.15 TLC plate analysis of the oligosaccharide content in intracellular (lanes 2-6) and extracellular (lanes 8-12) fractions of samples withdrawn from the culture of GD_{1a} and GT_{1a} producing NF17 strain. Standards are in lanes 1 and 7 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent. 79

Figure 4.1 ELISA protocol for the determination of antibody titer. 93

Figure 4.2 Binding of IgM (green square) and IgG (blue circle) antibody from immune sera obtained from mice immunized with GM_3-TT conjugate **34** to GM_3-BSA conjugate **40**. Each curve represents the average response of three mice. 94

Figure 4.3 Binding of IgG from immune sera against GM_3-TT conjugate **34** to BSA glycoconjugates **40** (GM_3, green square), **42** (lactose, red circle), **43** (glucose, black triangle), and **44** (GM_4, blue down triangle). Each curve represents the average response of three mice immunized with glycoconjugate **34**. 95

Figure 4.4 Protocol for determination of IC_{50} values. 96

Figure 4.5 Competitive ELISA assay for immune sera raised against GM_3-TT conjugate **34**. Synthetic oligosaccharides **30** (green square) and **46** (blue down triangle) inhibit binding of IgG to immobilized GM_3-BSA antigen **40**. IC_{50} values were determined to be 0.39 μM for **30** and 46.5 μM for **46**. 97

Figure 4.6 Binding of IgM (green square) and IgG (blue circle) antibody from immune sera obtained from mice immunized with GM_2-TT conjugate **35** to GM_2-BSA conjugate **41**. Each curve represents the average response of three mice. 99

Figure 4.7 Binding of IgG from immune sera against GM_2-TT conjugate **35** to BSA glycoconjugates **41** (GM_2, green square), **40** (GM_3, red circle), **42** (lactose, black triangle), **43** (glucose, blue down triangle) and **44** (GM_4, cyan diamond). Each curve represents the average response of three mice immunized with glycoconjugate **35**. 99

Figure 4.8 Competitive ELISA assay for immune sera raised against GM_2-TT conjugate **35**. Synthetic oligosaccharides **31** (green triangles), **47** (red down triangles), **30** (black square), **46** (cyan diamond), **18** (magenta cross), and **45** (blue star) inhibit binding of IgG antibodies to immobilized GM_2-BSA antigen **41**. IC_{50} values were determined to be 0.052 μM for **31** , 2.39 μM for **47**, 20.63 μM for **30**, 34.87 μM for **46**, 20.63 μM for **18** , and 526.69 μM for **45**. 100

Figure 4.9 Structure of the glycolipid haptens used in this study. 102

Figure 4.10 Binding of IgG from immune sera against GM_3-TT conjugates **58**, **59**, **34**, and **60** to BSA glycoconjugates **61** (green triangle), **62** (blue down triangle), **40** (black square), and **63** (magenta circle) respectively. Each curve represents the average response of five mice immunized with the respective glycoconjugates. 105

Figure 5.1 Targeting a glycoconjugate vaccine to dendritic cells using a bispecific fusion protein construct. 113

Figure 5.2 ELISA protocol for detecting bispecific fusion protein. 115

Figure 5.4 Structure of the target biotinylated antigens. 116

Figure 5.5 Humoral immune response against the biotinylated gangliosides **68** and **69**. ELISA plates were coated with GM_3- and GM_2-BSA **40** and **41** conjugates respectively. The optical density readings are for 1:1,000 serum dilution and are average responses of five mice. 120

Figure 5.6 Titration of mouse antisera derived from immunization with biotinylated GM_3- and GM_2 derivatives **68** and **69**. ELISA plates were coated with GM_3- and GM_2- BSA conjugates **40** and **41** respectively. Data are average responses of five mice. 121

Figure 5.7 Cellular immune response from mice immunized with biotinylated glycolipids **68** and **69**. Data are average responses of five mice. 122

List of Schemes

Scheme	Title	Page
Scheme 2.1	Synthesis of truncated azidosphingosine **1** and its selectively protected derivative 9.	44
Scheme 2.2	Synthesis of truncated lactosyl sphingosine **11**.	45
Scheme 2.3	Synthesis of lactosyl sphingosine **12**.	46
Scheme 2.4	Chemoenzymatic synthesis of GM_3 and GM_2 analogues **2** and **3**.	49
Scheme 2.5	Derivatization of GM_3 and GM_2 analogues with different acyl chains.	51
Scheme 2.6	Alternative method for the chemoenzymatic synthesis of GM_3 and GM_2 analogues.	52
Scheme 4.1	Preparation of tetanus toxoid and bovine serum albumin conjugates using an adipate linker.	89
Scheme 4.2	Preparation of bovine serum albumin glycoconjugates with squarate.	90
Scheme 4.3	Synthesis of S-octyl GM_3 derivative **46** and S-octyl GM_2 derivative **47**.	91
Scheme 4.4	Preparation of the GM_3-TT conjugates **58**, **59**, and **60**.	103
Scheme 4.5	Preparation of GM_3-BSA conjugates **61**, **62**, and **63**.	104
Scheme 5.1	Synthesis of GM_3 **50** and GM_2 **67** glycolipids.	117
Scheme 5.2	Biotinylation of GM_3 and GM_2 glycolipids.	118
Scheme 5.3	Synthesis of coupling reagent **71**.	124
Scheme 5.4	Synthesis of amino-fuctionalized GM_2 antigenic determinant.	125
Scheme 5.5	Synthesis of GM_2-acrylate derivative **76**.	125
Scheme 5.6	Synthesis of biotinylated glycopeptide **66**.	127

Abbreviations

α-2,3-SiaT	α-2,3-sialyltransferase
a	axial
A	alanine
Ab	antibody
Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
AcOEt	ethyl acetate
ADP	adenosine diphosphate
Ag	antigen
AgOTf	silver trifluoromethanesulfonate, silver triflate
Ala	alanine
AP	alkaline phosphatase
APC	antigen presenting cell
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATCC	American type culture collection
ATP	adenosine triphosphate
β-1,4-GalNAcT	β-1,4-*N*-acetylgalactosaminyltransferase
B-BSA	biotinylates bovine serum albumin
BCA:	bicinchoninic acid
B-GM_3	biotinylated GM_3
B-GM_2	biotinylated GM_2
bio	biotin
Boc	*tert*-butyloxycarbonyl
BSA	bovine serum albumin
BzCl	benzoyl chloride
c	*cis*

CD	cluster of differentiation
CDP	cytidine diphosphate
CFA	complete Freund's adjuvant
CgtA	*E. coli* clone expressing a β-1,4-*N*-acetylgalactosaminyl transferase from *C. jejuni*
CH_2Cl_2	dichloromethane
C. jejuni	campylobacter jejuni
CLEC4A	C-type lectin family 4 member A
CMP	cytidine monophosphate
CMP-Neu5Ac	cytidine-5'-monophospho-*N*-acetylneuraminic acid
CST-04	*E. coli* clone expressing an α-2,3-sialyltransferase from *C. jejuni*
Cst-II	α-2,3/2,8-sialyltransferase from *C. jejuni*
Cst-III	mutated α-2,3/2,8-sialyltransferase from *C. jejuni* which cannit use GM_1 as an acceptor
CTP	cytidine triphosphate
D	aspartic acid
DC	dendritic cell
DCC	*N,N'*-dicyclohexylcarbodiimide
DCIR	dendritic cell immunoreceptor
DC-SIGN	dendritic cell-specific intercellular adhesion moleule-3-grabbing non-integrin; aka CD209
DHB	2,5-dihydroxybenzoic acid
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-*N,N'*-dimethylaminopyridine
DMEM	Dulbecco's modified Eagle's medium
DMF	*N,N*-dimethylformamide
DMTST	dimethyl(methylthio)sulfonium trifluoromethanesulfonate
DTT	D/L-dithiothreitol
e	equatorial
E	glutamic acid

E. coli	*Escherichia coli*
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide
EDTA	ethylenediaminetetraacetic acid
EGC II	endoglycoceramidase II
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESI HRMS	electrospray ionization high resolution mass spectrometry
Et_3N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
Fruc-6-P	fructose-6-phosphate
GAM-HRPO	horseradish peroxidase-labeled goat anti-mouse antibody
G	glycine
Gal	galactose
GalNAcT	*N*-acetylgalactosaminyltransferase
GalT	galactosyltransferase
gCOSY	gradient coupling correlated spectroscopy
Glc	glucose
GlcN-1-P	glucosamine-1-phosphate
GlcN-6-P	glucosamine-6-phosphate
GlcNAc-6-P	*N*-acetylglusosamine-6-phosphate, 2-acetamido-2,6-dideoxy-6-phospho-D-glucose
GlmM	phosphoglucosamine mutase
GlmU	bifunctional acetyltransferase/uridylyltransferase
GlmS	*N*-acetyl-D-glucosamine-1-phosphate acetyltransferase
Gln	glutamine
Glu	glutamic acid
Gly	glycine

Gne	glucosamine(UDP-N-acetyl)-2-epimerase/ *N*-acetylmannosamine kinase
gTOCSY	gradient total correlation spectroscopy
h	hour
H_2O	water
HBr	hydrobromic acid
HBTU	*O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
hsp	heat shock protein
I	isoleucine
IAD	intramolecular aglycon delivery
IC_{50}	inhibitor concentration required to give 50% inhibition
IFA	incomplete Freund's adjuvant
IFN-γ	interferon gamma
Ig	immunoglobulin
Ile	isoleucine
IPTG	isopropyl β-D-1-thiogalactopyranoside
ivDde	1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl
K	lysine
KDN	3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid
KI	potassium iodide
KLH	keyhole limpet hemocyanin
L	leucine
LacY	lactose permease encoded by the Lac operon
LacZ	β-galactosidase encoded by the Lac operon

Leu	leucine
Lox-1	lectin-like oxidized low-density lipoprotein receptor
Lys	lysine
mAb	monoclonal antibody
MALDI-TOF	matrix assisted laser desorption ionization time of flight
ManNAc	*N*-acetyl-mannosamine, 2-acetamido-2-deoxy-6-D-mannose
ManNAc-6-P	*N*-acetyl-mannosamine-6-phosphate, 2-acetamido-2,6-dideoxy-6-phospho-D-mannose
MeOH	methanol
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
μmol	micromole
MK	monophosphate kinase
mL	milliliter
mmol	millimole
MMR	macrophage mannose receptor
$MnCl_2$	manganese chloride
MRC-1	mannose receptor C type 1
Ms	methanesulfonyl
MS	molecular sieves
MsCl	methanesulfonyl chloride
N	asparagine
$NaBH_4$	sodium borohydride
NagA	α-*N*-acetylgalactosaminidase
NagB	glucosamine-6-phosphate deaminase
$NaHCO_3$	sodium bicarbonate
$NaIO_4$	sodium periodate
NanA	sialic acid aldolase
NanE	*N*-acetylmannosamine-6-phosphate 2-epimerase
NanK	*N*-acetylmannosamine kinase
NanT	Sialic acid permease

NaOMe	sodium methoxide
Na_2SO_4	sodium sulfate
Neu	neuraminic acid
Neu5Ac	*N*-acetyl neuraminic acid, 3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid
Neu5Gc	*N*-glycolylneuraminic acid
NeuA	CMP-Neu5Ac synthetase
NeuB	Sialic acid synthetase
NeuC	GlcNAc-6-phosphate-2-epimerase
NHS	*N*-hydroxysuccinimide
NIS	*N*-iodosuccinimide
N. meningitides	*Neisseria meningitides*
NMK	nucleoside monophosphate kinase
NodC	chitooligosaccharide synthase
nst	α-2,3-sialyltransferase form *N. meningitidis*
NMR	nuclear magnetic resonance
NSY-05	cytidine-5'-monophospho-*N*-acetylneuraminic acid synthetase
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1
OmpT	omptins proteases
OtBu	*tert*-butyl ester
PBS	phosphate buffer saline
PBST	phosphate buffer saline containing Tween 20
Pi	phosphate
PK	pyruvate kinase
P_{Lac}	lactose promoter
PPase	pyrophosphatase
PPh_3	triphenylphosphine
PPi	pyrophosphate
ppm	parts per million
Pyr	pyridine

Q	glutamine
R	arginine
R_f	retention factor
RP-HPLC	reverse phase high performance liquid chromatography
rpm	rotations per minute
SATA	*N*-succinimidyl-*S*-acetylthioacetate
ScFv	single chain variable fragment
Ser	serine
SiaT	sialyltransferase
Sphing	sphingosine
trSphing	truncated sphingosine
t	*trans*
TACA	tumor associated carbohydrate antigen
TCA	tumor carbohydrate antigen
T_C lymphocyte	cytotoxic T lymphocyte
TFA	trifluoroacetic acid
TfOH	triflic acid
THF	tetrahydrofuran
T_H lymphocyte	helper T lymphocyte
Thr	threonine
TLC	thin layer chromatography
TMB	3,3′,5,5′-tetramethylbenzidine
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TOF	time of flight
tR	retention time
Tris HCl	TRIZMA hydrochloride
TRITC	tetramethylrhodamine isothiocyanate
trSphing	truncated sphingosine
Trt	trityl
TSCA	tumor specific carbohydrate antigen
UDP	uridine 5'-diphosphate

UDP-Gal	uridine 5'-diphosphogalactose
UDP-GalNAc	uridine 5'-diphospho-*N*-acetylgalactosamine
UDP-Glc	uridine 5'-diphosphoglucose
UDP-GlcNAc	uridine 5'-diphospho-*N*-acetylglucosamine
UTP	uridine 5'-triphosphate
UV	ultraviolet

Chapter 1
Introduction: Ganglioside Conjugate Vaccines for Tumor Immunotherapy

1.1 Carbohydrates in biological systems: Their structure, biosynthesis and significance

Eukaryotic cells are characterized by the display of a dense array of glycans at their surfaces. It is thus not surprising to find these macromolecules act as key elements in important molecular recognition events.[1,2] Oligosaccharides at the surface of eukaryotic cells mediate *cis* and *trans* recognition processes occurring during fertilization,[3,4] embryogenesis,[5] metastasis, neuronal development,[6,7] inflammation,[8] immune response,[9] cell proliferation, and pathogen recognition.[10] Furthermore, by their intrinsic properties, glycans serve a structural role acting as a scaffold for the organization of other glycans and proteins of the extracellular matrix. Even though great advances have been made in understanding these mechanism over the past 20 years, the specific details of these interactions are often sketchy. Glycobiology aims at elucidating the details of these molecular recognition events and modulating the outcomes of these interactions.[11]

1.1.1 Glycoproteins

Oligosaccharides are presented at the cell surface as glycoconjugates linked either to proteins or lipids. Two types of glycoproteins can be distinguished according to the type of linkage between the glycan reducing sugar and the amino acid.[12,13] *N*-linked glycoproteins are characterized by a common pentasaccharide core attached to the amide nitrogen of asparagine residues *via* an *N*-glycosidic bond (Figure 1.1)[12]. Several other monosaccharide units extend from this core generating the wide variety of species found in eukaryotic cells. Despite their diversity, all *N*-linked glycoproteins are assembled through a common pathway. Assembly of *N*-linked glycoproteins is initiated in the endoplasmic reticulum (ER) where a series of glycosyltransferases catalyse the formation of a lipid-linked oligosaccharide precursor. This oligosaccharide is then transferred enbloc to asparagine residues on the nascent polypeptide chain. Glycosylated asparagine residues are almost invariably found in the consensus sequences Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except proline, although the presence of this sequence is not enough to ensure glycosylation.[14] After extensive processing by exoglycosidases and transfer to the Golgi apparatus, the glycan is futher elaborated by different glycosyltransferases to give a heterogeneous assembly of glycoproteins necessary for cell function.

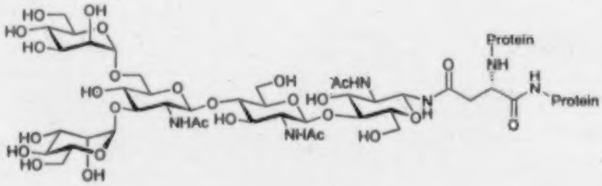


Figure 1.1 *Core structure of N-linked glycoproteins*

N-Linked glycans share a common protein-glycan linkage and fall into relatively few structural classes, reflecting a common biosynthetic pathway that only diverges in its late stages. In contrast, *O*-linked glycans are build on different protein-glycan linkages in which *N*-acetylgalactosamine, fucose,[15] *N*-acetylglucosamine,[16] mannose,[17] xylose, or galactose can be attached to either serine, threonine, or hydroxylysine residues. The heterogeneity of *O*-linked glycoprotein stems from the very nature of their biosynthesis. As opposed to *N*-linked glycosylation, there is no target sequence that defines the sites of *O*-linked glycosylation. The biosynthesis of *O*-linked glycans typically occurs post-translationally in the Golgi apparatus via the non-templated stepwise addition of monosaccharide units to the protein. Despite their heterogenicity, *O*-linked glycans are typically constructed around five relatively simple common structural motifs (Figure 1.2) that cluster at the surface of membrane proteins to form large hydrophilic domains in mucins and proteoglycans at cell and tissue interfaces.

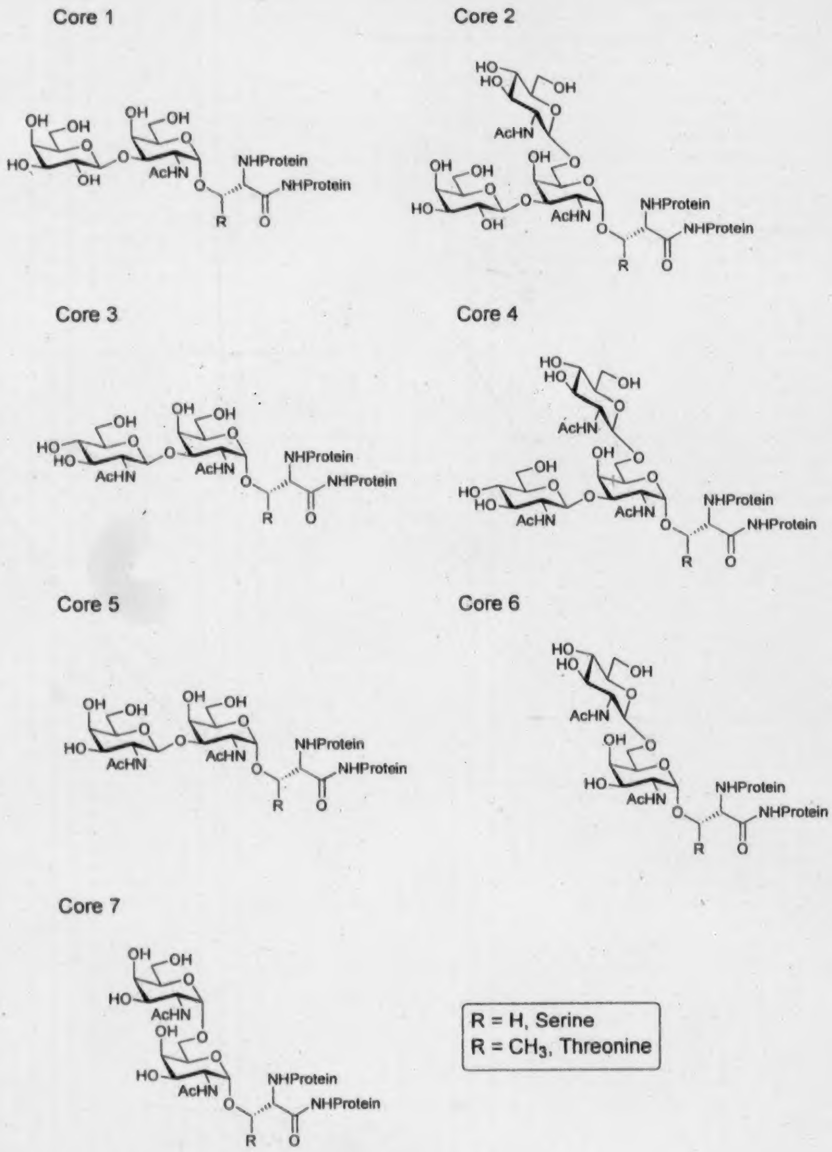

Core 1
Core 2
Core 3
Core 4
Core 5
Core 6
Core 7
R = H, Serine
R = CH$_3$, Threonine

Figure 1.2 *Core structure of O-linked glycoproteins.*

1.1.2 Glycolipids

In addition to being presented at the cell surface as glycoproteins, glycans can also be presented at the cell surface by attachment to the head group of lipids. Glycolipids fall into two distinct structural categories according to the type of their lipid portion.[12]

Glycosphingolipids are built around a ceramide core attached to either a glucose or a galactose moiety.[18, 19] Their biosynthesis is initiated in the endoplasmic reticulum where a glucose or a galactose residue is transfered from the corresponding sugar nucleotide donor to the pre-formed ceramide. The basic glycosphingolipid structure (Figure 1.3) is then further elaborated by the addition of other carbohydrate residues or sulfatides in the Golgi apparatus. The final products are then directed to the plasma membrane where they tend to cluster in specialized membrane domains to accomplish their function in cell-cell interactions.[20] In contrast to glycosphingolipids, the glycan of glycophospholipids is not directly exposed to the extracellular environment. In glycophospholipids, the glycan acts as a hinge for anchoring proteins to the cell surface. Different structures of glycophospholipids exist but they all share common features (Figure 1.3B). All glycophospholipids are centered around a phosphatidylglycerol core expanding into a mannose-rich glycan. The glycan bridges the protein and the lipid by providing attachment points for both components. The glycan is attached at one end to the inositol head group of the lipid *via* a β-(1→6) linkage to a reducing glucosamine residue. At the other end, the core glycan structure is modified at a mannose residue by a phosphodiester-linked ethanolamine residue. This ethanolamine residue provides a free amino group that readily condenses with the C-terminal carboxyl group of the protein. This basic structure can undergo further modifications. [12] As for *N*-linked glycoproteins, the biosynthesis of glycosphingolipid occurs in the endoplasmic reticulum and is transferred as a pre-assembled unit to the protein. The assembled glycophospholipid-modified protein is then directed to the plasma membrane to effect its function in the cell.

1.1.3 Significance of carbohydrate interactions

Over the last 20 years, glycobiology has slowly changed the traditional paradigm in which homotypic protein-protein interactions controlled cellular recognition events.[21] It is now recognized that glycoconjugates at the cell surface are important mediators of cellular events through multiple carbohydrate-carbohydrate and carbohydrate-protein binding events.[22] Glycans have inherently low affinity for the proteins or other glycans involved in the binding and rely heavily on multivalency for the strength of their interactions.[23] Multivalent binding (the simultaneous association of two or more receptors with their ligands) results in an interaction of enhanced avidity and specifity. It thus provides a platform for the carbohydrate to achieve binding of sufficient strength and specificity for the relay of biological information.[24]

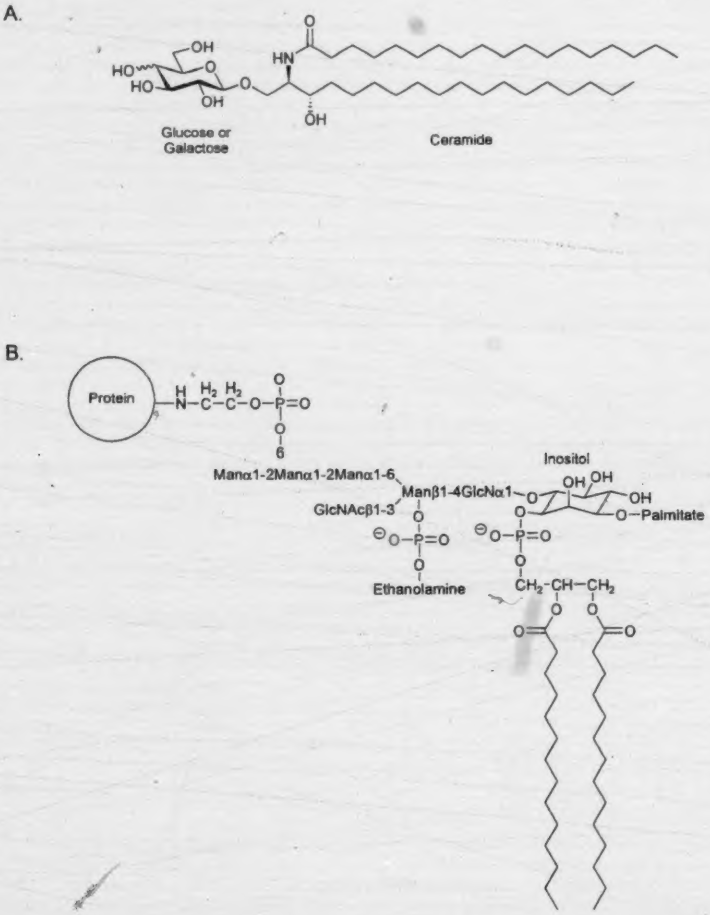

A.
HO
HO
OH
OH
O
HN
O
OH
Glucose or Galactose
Ceramide
B.
Protein
H
N
H_2
H_2
P
6
Manα1-2Manα1-2Manα1-6
Manβ1-4GlcNα1
GlcNAcβ1-3
Inositol
OH
OH
OH
O—Palmitate
Ethanolamine
CH_2-CH-CH_2

Figure 1.3 *Basic structure of (a) glycosphingolipids and (b) glycophospholipids.*

1.2 Carbohydrate synthesis

1.2.1 Chemical methodologies

The details of carbohydrate-mediated events must still be unraveled and are the focus of many investigations. The study of carbohydrate mediated events is complicated by the inherent nature of this biopolymer and its interactions. Contrary to other biopolymers, carbohydrates are frequently non-linear and their subunits combine to form a multitude of positional and stereochemical isomers resulting in microheterogeneity. This microheterogeneity combined with the scarcity of several oligosaccharides complicates access to important structures in pure form from cellular sources. Due to these difficulties, many studies of carbohydrate-mediated events have relied on indirect methods such as anti-carbohydrate antibodies to localize and characterize these processes. However, a complete image would not be possible without defined synthetic ligands to probe these interactions.

1.2.1.1 General considerations

Oligosaccharides are formed from an ensemble of monosaccharide units joined to each other *via* acetal or ketal linkages. The main challenge of oligosaccharide synthesis is to form these linkages in a regio- and stereo-controlled manner.[25] A typical glycosylation reaction involves the condensation of a fully protected glycosyl donor which bears a leaving group at its anomeric position with an appropriately masked glycosyl acceptor.[26] Activation of the glycosyl donor by a promoter followed by departure of the leaving group leads to the formation of a oxocarbenium ion intermediate which can be attacked from either the top or bottom face by the glycosyl acceptor to form the glycosidic linkage. (Figure 1.4).

1.2.1.2 Glycosyl donors

Different types of glycosyl donors have been applied to the synthesis of glycosides.[27] The classical Knoenigs and Knorr method utilizes glycosyl halides as donors, taking advantage of their ease of synthesis and activation with heavy metal salts. However, their instability and the strongly acidic conditions required for their formation greatly limit their synthetic utility.[28] More modern glycosyl donors include thioglycoside and trichloroacetimidate donors which are now used in the majority of syntheses.

Thioglycosides are extremely versatile glycosyl donors.[29] They are stable to most protecting group manipulations and can therefore be introduced at an early stage of the synthesis, acting both as a protecting group for the anomeric position and as a leaving group upon activation. Furthermore, they can be easily converted to more reactive donors when required for more difficult glycosylations. Trichloroacetimidates have also found many uses in the construction of oligosaccharides as they can be introduced under mild conditions. [30]

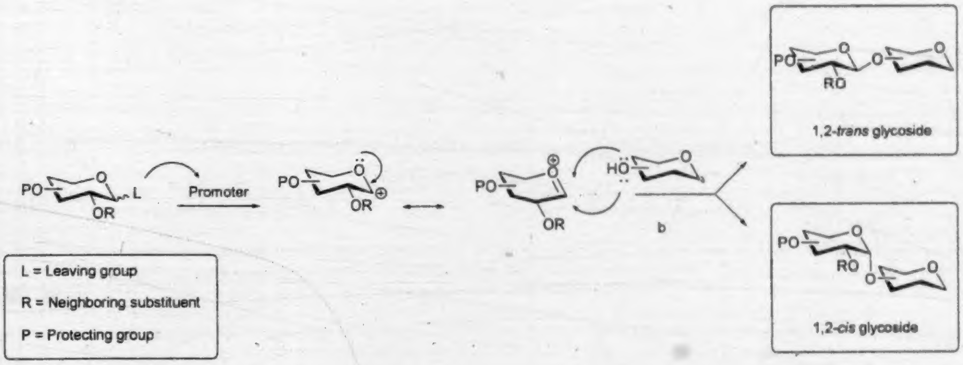


Figure 1.4 *General mechanism of glycosylation reactions.*

The orientation of the OH group in relation to the anomeric substituent characterizes the newly formed glycosidic bond. Four classes of glycosidic bonds can be distinguished: 1,2-trans-α, 1,2-trans-β, 1,2-cis-α, and 1,2-cis-β (Figure 1.5). [31] A general strategy can be applied to the synthesis of each class of glycosidic bond, each of which will be discussed in the following sections.

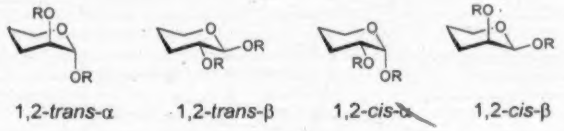

RO
OR
1,2-trans-α
OR
OR
1,2-trans-β
RO
OR
1,2-cis-α
RO
OR
1,2-cis-β

Figure 1.5 *Types of glycosidic linkages.*

1.2.1.3 Formation of the 1,2-*trans*-α and 1,2-*trans*-β linkages

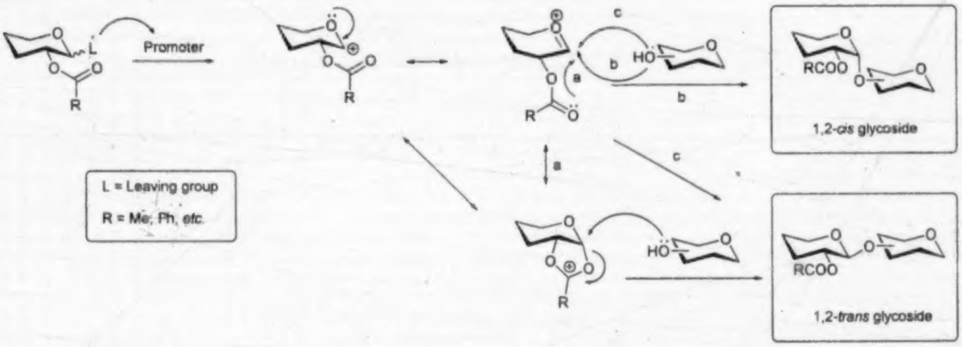

L
R
Promoter
R
c
b
HO
a
R
b
RCOO
1,2-cis glycoside
a
c
L = Leaving group
R = Me, Ph, etc.
HO
R
RCOO
1,2-trans glycoside

Figure 1.6 *Neighboring group participation in the formation of 1,2-trans glycosidic linkages.*

The formation of 1,2-*trans*-α and 1,2-*trans*-β linkages rely upon the presence of a participating group at the C-2 position that is able to direct the incoming acceptor in a *trans* orientation (Figure 1.6).[26, 32-34] As previously mentioned, activation of the anomeric leaving group generates an oxocarbenium intermediate. Neighbouring group participation of a 2-*O*-aceyl protecting group results in the formation of the more stable acetoxonium ion. Subsequent attack of the glycosyl acceptor at the anomeric centre results in the formation of the 1,2-trans glycoside. As the acetoxonium intermediate is bicyclic, attack of the acceptor is directed to the opposite face of the ring generating the stereoselectivity of the reaction. It is also possible for the glycosyl acceptor to attack the center of the dioxolane ring at C-2 to produce a transient orthoester which usually rearranges under the acidic reaction conditions to yield the desired product.

Although this method to form 1,2-trans glycosidic bonds is very effective, it is not always possible to introduce a participating group at the C-2 position. Several reports have shown that it is possible to generate an equatorial 1,2-trans bond in the absence of a participating group using a participating solvent.[35,36] This method was of particular interest to establish sialosyl linkages [37] and will be further discussed in Section 2.1.2.

1.2.1.4 Formation of 1,2-*cis*-α linkages

The formation of 1,2-cis linkages are generally more challenging than their 1,2-trans counterparts since the stereoselectivity of their formation cannot be as well controlled. Methods for the synthesis of 1,2-cis-glycosides employ a glycosyl donor with a non-participating group, generally an alkyl group, at the C-2 position in a non-polar solvent and rely on the relative energies of the transition states involved in the formation of α- and β-glycoside to direct the stereoselectivity of the reaction.[26] Major advances were made in this area in the 1970's with the development of the *in situ* anomerization procedure (Figure 1.7).[38,39] Accordingly, reaction of a glycosyl bromide donor with an acceptor in the presence of tetrabutylammonium bromide gives almost exclusively the α-glycoside product. Tetrabutylammonium bromide catalyses a rapid equilibration between the α- and β-halides. This equilibrium is shifted towards the α-anomer as the anti-periplanar arrangement of the ring oxygen electrons and the geminal polar bond stabilize this anomer (anomeric effect). On the other hand, attack of the incoming nucleophile is more favored for the β-anomer as the anti-periplanar arrangement of the ring and incoming nucleophile oxygen atom lone pairs lowers the energy of the transition state. Glycosylation is thus proposed to proceed *via* the β-bromide in an S_N2-type pathway to give the desired α-glycoside (Figure 1.7). This procedure was also extended to more modern donors.[40]

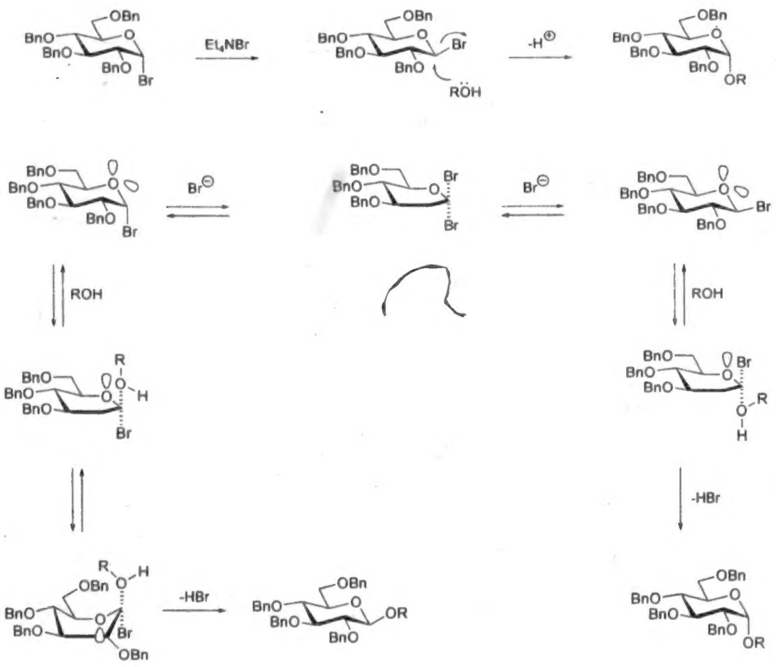


Figure 1.7 *Formation of 1,2-cis-α linkages.*

1.2.1.5 Formation of 1,2-cis-β linkages

The 1,2-cis-β linkage is one of the most difficult bonds to establish in carbohydrate chemistry as neither anchimeric assistance nor the anomeric effect can be used to favor formation of this glycosidic bond. Several strategies have been developed to access, with some success, 1,2-cis-β glycosides. These methods include the use of solid-supported promoters,[41] inversion of the C-2 position,[42,43] oxidation/reduction of the easily prepared β-glycoside,[44] and intramolecular aglycon delivery.[45,46] These methods tend to be specialized for individual cases and a detailed discussion of their mechanisms is beyond the scope of this overview.

Carbohydrate chemistry has witnessed tremendous progress since the development of the first glycosylation reaction by Fisher. A variety of glycosyl donors, protecting groups and methodologies have been developed allowing chemists to tackle impressive targets. Recently, Lowary *et al.* achieved the impressive synthesis of a 22-residue oligosaccharide.[47] However, despite these successes, the chemical synthesis of oligosaccharides is still challenging as the control of regioselectivity involves numerous protecting group manipulations and the application of glycosylation methodologies is case dependent.

1.2.2 Biological approaches to the synthesis of oligosaccharides

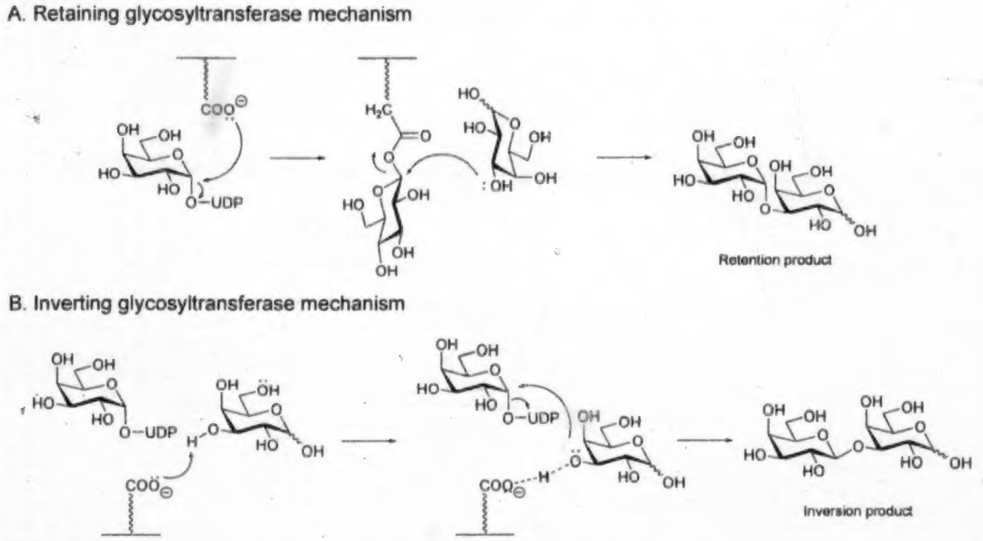
Biological approaches to the synthesis of oligosaccharides greatly ease the production of oligosaccharides, allowing for their regioselective and stereoselective production without the use of protecting groups. Four approaches, including the use of glycosyltransferases, glycosidases, glycosynthases, and metabolic engineering of microorganisms, have emerged in the recent years as powerful means to prepare oligosaccharides. These tools are particularly useful for the synthesis of glycosidic linkages that are difficult to establish such as α-sialosyl, α-fucosyl, and β-mannosyl linkages.

1.2.2.1 Glycosyltransferases

Glycosyltransferases catalyze the transfer of a monosaccharide unit from an activated donor to an acceptor in a highly specific manner.[33] These enzymes are classified according to the nature of the sugar donor they use for the formation of the glycosidic bond. Glycosyltransferases of the Leloir pathway are involved in the biosynthesis of most mammalian glycoconjugates and oligosaccharides and utilize sugar nucleotides as donors whereas glycosyltransferases of the non-Leloir pathway use sugar-phosphate derivatives as donors.[48-50] Glycosyltransferases catalyse the formation of glycosides *via* two different mechanisms leading to product with either retention or inversion of anomeric stereochemistry (Figure 1.8).[33] The retaining mechanism involves a double displacement in which a glycosyl-enzyme intermediate is first formed with concomitant release of a nucleotide diphosphate molecule. The glycosyl-enzyme intermediate is then attacked by the glycosyl acceptor resulting in the release of glycoside with inversion of configuration with respect to the donor. In contrast, a single displacement step occurs in inverting glycosyltransferases in which the acceptor acts as a direct nucleophile to attack the glycosyl donor at the anomeric position. Glycosyltransferases have found many uses in the synthesis of oligosaccharides allowing access to large quantities of structures with minimal synthetic transformations. However, several of their features limit their application in oligosaccharide synthesis. Glycosyltransferases are highly specific in the reactions they catalyze, tolerating only minor modifications in either the donor or acceptor.[51-53] This greatly limits the breadth of structures that can be accessed using this methodology. Furthermore, glycosyltransferases are difficult reagents to obtain. As membrane proteins, their expression in bacterial systems tends to be hampered by poor solubility, their purification is difficult and they tend to be unstable on prolonged storage. The effort to obtain these enzymes combined with the high cost of sugar-nucleotide donors renders the process relatively expensive. *In situ* regeneration of cofactors alleviate the cost of the sugar nucleotide by recycling the nucleotide cofactors using an inexpensive high energy molecule such as phosphoenolpyruvate.[54-56] The development of these systems requires expansive efforts that can only be practical for large-scale preparations. Glycosyltransferase-catalyzed preparation of oligosaccharides constitutes a very powerful approach to the synthesis of natural structures, especially those containing difficult linkages. Despite its advantages, the process can still be quite expensive and the oligosaccharide derivatives that can be obtained by this approach are limited. Thus a broader and more cost effective approach is still being sought.[57]

1.2.2.2 Glycosidases

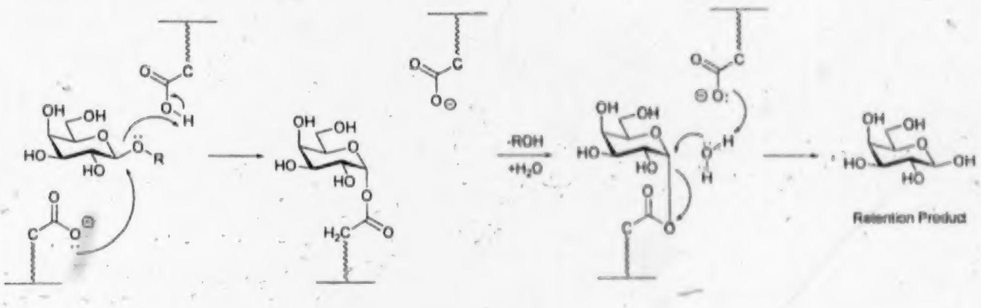
The scarcity of glycosyltransferases and difficulty associated with their preparation motivated the exploration of glycosidases as catalysts for glycoside formation. Glycosidases usually catalyze the hydrolysis of glycosidic bonds but their reverse reaction can be exploited for the formation of glycosides. By analogy with glycosyltransferases, glycosidases catalyze the hydrolysis of glycosides *via* two mechanisms: One leading to retention of anomeric configuration and the other resulting in inversion (Figure 1.9).[58-60] The reaction of inverting glycosidase involves a single displacement of a leaving group by water assisted by general acid/base catalysis effected by a pair of strategically placed carboxylic acid residues in the active site. The active site of retaining glycosidases also contains two strategically located carboxyl groups that effect catalysis, but one of them acts as a general acid whereas the other acts as a nucleophile. The mechanism of retaining glycosidases first involves protonation of the glycosidic oxygen by one carboxyl group while the second carboxyl residue performs a nucleophilic attack at the anomeric carbon resulting in the formation of an oxocarbenium ion-like transition state. Displacement of the anomeric substituent results in a glycosyl-enzyme intermediate with an anomeric configuration opposite to that of the initial substrate. Under normal conditions, this intermediate is trapped by water to generate the hydrolyzed product. However, under appropriate conditions, this glycosyl-enzyme intermediate can be intercepted by another nucleophile to generate a transglycosylation product with the same anomeric configuration as the initial substrate. While being significantly more accessible than glycosyltransferases, glycosidase-catalyzed reactions are typically less regioselective and proceed in low to moderate yield, since the transglycosylation product can be hydrolyzed by the enzyme.[33]


A. Retaining glycosyltransferase mechanism

B. Inverting glycosyltransferase mechanism

Figure 1.8 *Mechanism of (a) retaining and (b) inverting glycosyltransferases.*

A. Retaining glycosidase mechanism



B. Inverting glycosidase mechanism

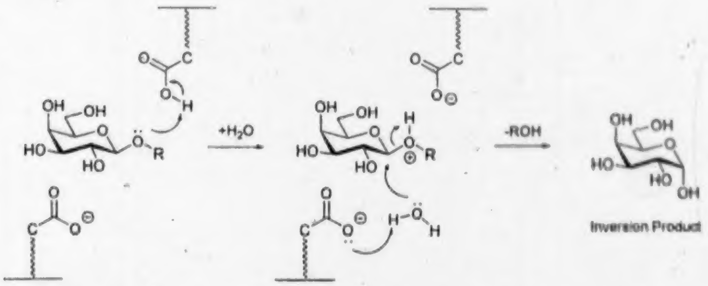


Figure 1.9 *Mechanism of (a) retaining and (b) inverting glycosidases.*

It has been shown that transglycosylation yields of retaining glycosidases can be greatly improved by rational mutation of the enzyme. Elegant work by Withers *et al.* have demonstrated that mutation of the active site nucleophile to a smaller amino acid residue such as alanine completely inactivates the hydrolytic activity of the enzyme by preventing the formation of a covalent glycosyl-enzyme intermediate (Figure 1.10).[59] If this mutant enzyme is presented with a reactive donor of opposite anomeric configuration, the enzyme catalyses its glycosylation of a sugar acceptor. Such mutant enzymes are called glycosynthases. Glycosyl fluorides are typically used as the donor due to their solubility and stability under the reaction conditions.[61] Contrary to glycoside preparation with glycosidases or transglycosidases, the product formed cannot be hydrolyzed by the mutant enzyme and high yields of products can be obtained with remarkable catalytic efficiency.[58,59]

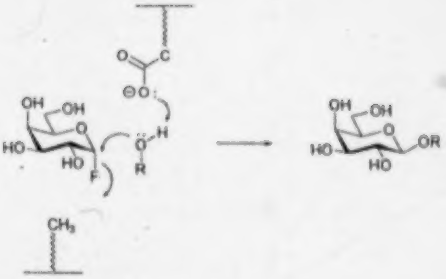


Figure 1.10 *Mechanism of glycosynthases.*

1.2.2.3 Oligosaccharide synthesis by metabolically engineered bacteria

The production of oligosaccharides by enzyme catalysis has been limited to small-scale synthesis as significant effort is required to produce large quantities of the catalyst in pure form. However, in the last 10 years, the use of metabolically engineered micro-organisms has emerged as a tool for the low-cost production of considerable amounts of complex carbohydrates.[62,63] This approach relies on using specially engineered bacteria or yeast cells to produce oligosaccharides in high yield without the need for enzyme isolation or expensive precursors. The glycosylation reactions are performed by whole cells overexpressing the genes encoding the appropriate glycosyltransferases and sugar-nucleotide biosynthesis enzymes leading to the accumulation of the desired products in the cell. This method has afforded the efficient production of impressive quantites of complex sugars including gangliosides, tumor associated antigens[64-69] and chitooligosaccharides[70] and promises to become an important tool for carbohydrate chemists.

1.3 Carbohydrates in drug discovery and development

The prevalent role of carbohydrates in numerous physiological events presents an avenue for therapeutic interventions in a wide range of diseases including cancer, malaria, HIV, tuberculosis and influenza.[71,72] Given the central role of carbohydrates in a wide range of biological processes, it may appear surprising that few carbohydrate-based drugs are on the market. Successful carbohydrate-based therapeutic agents include the anti-influenza drug Tamiflu; an α-amylase inhibitor, acarbose, used in the treatment of diabetes; the anticoagulant heparin; and prophylactic vaccines against bacterial infections.[72] The difficult access of oligosaccharides in pure form by isolation from natural sources or chemical synthesis combined with the difficulties associated with the characterization of polysaccharides have hampered the study of carbohydrate-mediated processes and by the same token the development of therapeutics. The design and development of glycotherapeutics falls into four different therapeutic strategies which include: Inhibition of carbohydrate processing enzymes that are either up-regulated or present only in the disease state, interference with carbohydrate-carbohydrate or carbohydrate-protein interactions, immunization with a carbohydrate antigen and exploitation of carbohydrate-protein binding specificity in drug delivery. Recent advances in synthetic methodologies and techniques for analysis of glycoconjugate composition have opened new doors for understanding molecular regognition and signaling events mediated by carbohydrates. Continued study of these processes will certainly provide insight into the design of new therapeutics.

1.4 Conjugate Vaccines

One of the main successes of modern medicine certainly lies in the development of prophylactic vaccines, which confer protection against a wide range of infectious agents. Carbohydrates play a central role in induced immunity as the antibodies generated by these vaccines often recognize glycan structures at the surface of these micro-organisms.

Carbohydrates are particularly attractive targets for vaccine development. Contrary to other biomolecules, their localization to the outer leaflet of the cell membrane renders them accessible to the immune system. Typical vaccination strategies rely on adaptive immunity to recognize and selectively eliminate specific foreign antigens.[73] The interplay of two major groups of cells, T lymphocytes and antigen presenting cells, is essential for the success of this approach (Figure 1.11). The first step of adaptive immunity involves the recognition of a foreign antigen by antigen presenting cells. Following endocytosis, the antigen is processed in a specific pathway resulting in its proteolytic degradation. If the antigen is a protein, this process generates small peptide fragments for T_H-cell activation. These T cell peptides are presented at the surface of the antigen presenting cell in association with an MHC molecule. Two classes of MHC molecules are used by the immune system to present antigens and direct the result of the response. Depending on its origin, an antigen can be either presented on a MHC class I or MHC class II molecule. Intracellular antigens are generally presented on MHC class I molecules and activate the cellular arm of the immune system resulting in direct attack of infected or cancer cells by cytotoxic T (T_C) lymphocytes. This is in contrast to extracellular antigens which are presented on MHC class II molecules. The antigen in complex with an MHC class II is recognized by a T cell receptor (TCR) at the surface of helper T (T_H) lymphocytes. This recognition event activates T_H cells. The activated T_H cells release cytokines, which in turn stimulates proliferation of antigen-specific B-lymphocytes. During this clonal expansion, B-lymphocytes cease the production of antibodies of the IgM subtype and start producing IgG antibodies in a process called isotype switching. The maturing B-lymphocytes also undergo affinity maturation whereby repeated mutations in their Ig hypervariable domain ultimately result in the selection of the B-cells clones secreting antibodies of higher affinity. The selected cells finally mature into IgG-secreting plasma cells or memory cells which can, upon re-exposure to the same antigen, rapidly induce a secondary reponse of higher amplitude.

Carbohydrates are generally poor immunogens, generating low levels of low-affinity IgM antibodies as they are incapable of activating T lymphocytes. These T_H-independent responses arise from the cross-linking of immunoglobulin (Ig) at the surface of B-lymphocytes by polysaccharides. While this primary response is efficient at activating innate processes, it fails to induce the immunological memory that provides long-lasting immunity against an antigen.

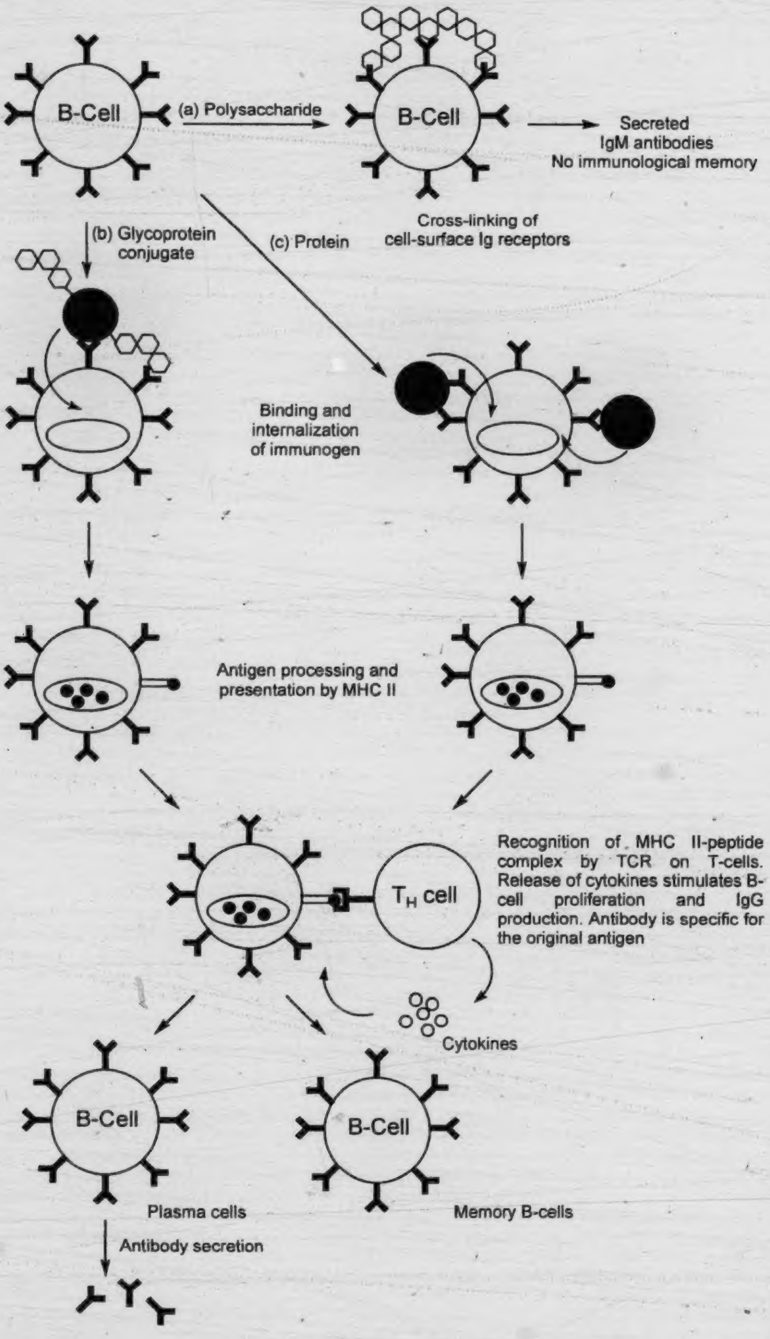

B-Cell
(a) Polysaccharide
B-Cell
Secreted
IgM antibodies
No immunological memory
Cross-linking of
cell-surface Ig receptors
(b) Glycoprotein
conjugate
(c) Protein
Binding and
internalization
of immunogen
Antigen processing and
presentation by MHC II
T_H cell
Recognition of MHC II-peptide complex by TCR on T-cells. Release of cytokines stimulates B-cell proliferation and IgG production. Antibody is specific for the original antigen
Cytokines
B-Cell
B-Cell
Plasma cells
Memory B-cells
Antibody secretion

Figure 1.11 *Humoral immune response to carbohydrate antigens.*

The T_H independent nature of carbohydrate antigens can be reversed by conjugation to an immunogenic carrier protein. If appropriately conjugated and administered in conjunction with an adjuvant, carbohydrates are capable of inducing good levels of protective antibodies and stimulating immunological memory (Chapter 4). A conjugate vaccine induces an enhanced response against a carbohydrate antigen by providing peptide fragments for activation of T_H-lymphocyte. The mechanism of its action is two-fold. Initially, the carbohydrate epitope is recognized by the immunoglobulin receptors of specific B-lymphocytes. Cross-linking of the cell surface immunoglobulin receptors by the carbohydrate induce internalization of the glycoconjugate and its degradation into peptide fragments for presentation to T_H lymphocytes. Recruitment of T lymphocyte help then results in the production of IgG antibodies specific for both the carbohydrate and protein component of the glycoconjugate and generation of immunological memory. Conjugate vaccines based on bacterial polysaccharides coupled to an immunogenic carrier protein have been quite successful in the clinic, providing protective immunity against infections with *Steptococcus pneumoniae*, *Neisseria meningitides*, and *Haemophilius influenza*. Recent investigations utilizing fully synthetic oligosaccharides provides hope for the development of new vaccines that will be useful in the treatment and prevention of cancer, bacterial and viral infections, and tropical diseases.

1.5 Tumor Immunotherapy

1.5.1 Tumor antigens

The transformation of normal cells to cancerous cells is associated with a disruption in the glycans displayed at their surface.[74,75] Malignant cells are commonly characterized by the expression of a distinct pattern of glycoproteins and glycolipids which differentiate them from normal cells. This abnormal glycosylation pattern arises from the aberrant function of glycosyltransferases and glycosidases involved in the biosynthesis and processing of these glycans. As a result, some glycan structures are overexpressed at the surface of cancer cells, others are underexpressed and some new structures make their appearance.[76] Histochemical studies have shown that both glycolipids and glycoproteins can be associated with tumors (Figure 1.12).[77] These tumor antigens are proposed to mediate the abnormal behavior of cancerous cells such as enhanced growth, invasiveness, and metastasis, possibly *via* their influence on cell adhesion.[76] Two types of tumor antigens can be distinguished. Tumor associated carbohydrate antigens (TACAs) refer to glycan structures that are found in normal tissues but which are found in much higher concentration on tumor cells. A lesser number of tumor specific carbohydrate antigens (TSCAs) have been identified. TSCAs are molecules that are not normally expressed on healthy cells, expressed in immune privileged sites, or expressed only during development.

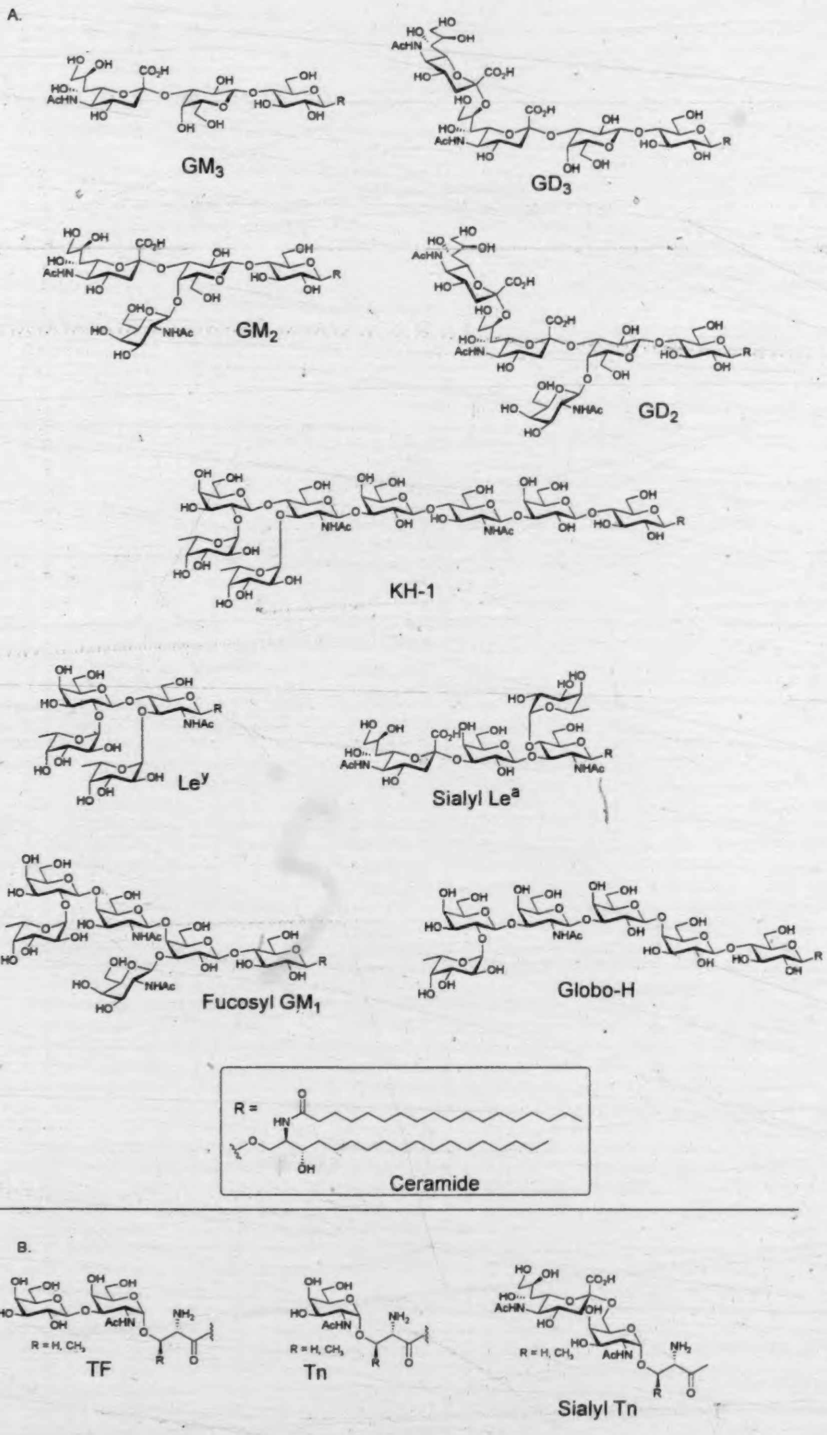

A.
GM$_3$
GD$_3$
GM$_2$
GD$_2$
KH-1
Le^y
Sialyl Le^a
Fucosyl GM$_1$
Globo-H
R =
Ceramide
B.
R = H, CH$_3$
TF
Tn
Sialyl Tn

Figure 1.12 *Examples of tumor associated carbohydrate antigens. Glycolipids are depicted in part (a) and glycoproteins in part (b).*

1.5.2 Gangliosides in tumor immunotherapy

The development of methods to enhance immunological recognition of tumor antigens and induction of immunity *in vivo* is particularly challenging due to the difficulties associated with breaking the body's immune tolerance.

Gangliosides comprise a group of over 30 glycosphingolipids present at the surface of normal cells in specialized domains of the plasma membrane. Gangliosides contain at least one sialic acid residue linked *via* a α-(2→3) or an α-(2→6) glycosyl linkage to a galactosyl derivative. Ganglioside structures are generally named using the Svennerholm nomenclature[82] which describes them in terms of both the number of sialic acid residues present in the structure and their relative mobility on silica gel thin layer chromatography. The prefix G is followed by a letter designating the number of sialic acid residues: M for mono, D for Di, T for tri and Q for tetra. This letter is in turn modified by a subscript number which indicates the relative migration of the ganglioside on a TLC plate within its category. For example, GM_3 contains a single sialic acid residue and is the third most polar monosialoside.

Alterations in ganglioside expression in association with tumor transformation were first reported in 1966 on brain tumors[83] and have since been observed in several other tumor tissues.[84,85] It has been proposed that gangliosides may be involved in many biological events associated with tumors, including cell growth, migration, invasion, and formation of metastases.[86] The overexpression of gangliosides in tumor cells combined with their role in various events associated with cancerous behavior make them attractive targets for tumor immunotherapy. The efficiency of a particular immunotherapy strategy is dependent on several factors, including the availability, concentration and density of the antigen, its ability to raise an immune response, the affinity of the induced antibodies for the antigen, and the antibody binding ratio between tumor and normal cells.[86] Gangliosides are particularly suited as a basis for tumor immunotherapy. Their localization on the cell surface makes them accessible for recognition by the effector molecules of the immune system. Furthermore, gangliosides are widely expressed in high concentrations on malignant cells derived from a large population of cancer patients. Despite these advantages, the development of ganglioside-based tumor immunotherapy presents several challenges. In particular, as carbohydrate antigens, gangliosides tend to be poorly immunogenic and are incapable of generating a cytotoxic T lymphocyte response to effect the clearance of tumor cells.

In an ideal situation, a cancer vaccine would stimulate both the cellular and humoral branches of the immune system to effect the clearance of tumor cells.[87] Cytotoxic T lymphocytes are able to directly destroy tumor cells *via* apoptosis or perforin-mediated lysis. While these processes are very efficient at eliminating tumor cells, humoral responses still have a place in tumor immunotherapy. Antibodies are particularly suited for elimination of circulating tumor cells and micrometastases.[88] Indeed, patients who present natural antibodies to tumor carbohydrate antigens or have been passively administered such antibodies have a better prognosis than patients in which no anti-tumor antigen antibodies could be detected.[89,90] Thus, the generation of sufficient antibody titers against tumor carbohydrate antigens has the potential of revolutionize cancer treatment.

Initial attempts to establish an immune response to tumor carbohydrate antigens relied on immunization with autologous or allogenic tumor cells and their lysate.[91-93] These immunizations resulted in a strong humoral response against different antigens. It was observed, however, that a response to only two antigens: GM_2 and GD_2 was consistent among the patients tested.[94] In an effort to create a more defined therapy, mice and patients were administered a purified sample of GM_2. Gangliosides alone were found to be non-immunogenic as no antibodies against the immunogen were detected. Immunization with a combination of the ganglioside and an adjuvant afforded slightly better results generating low titers of IgM antibodies.[95,96] These results were not surprising as gangliosides are endogenous antigens to which the immune system has developed tolerance. Approaches that included gangliosides in the context of immunogenic foreign antigens for presentation to the immune system were much more successful. Administration of a glycoconjugate consisting of a ganglioside attached to an immunogenic protein coupled with a potent adjuvant generated moderate titers of IgG antibodies in several clinical trial studies.[97, 98] However, for this therapy to be viable, the immunogenicity of these constructs must be significantly enhanced.[99]

1.6 Scope of the project

Preliminary reports have suggested that anti-ganglioside antibodies might bind with higher affinity to gangliosides with longer fatty acid chains.[87,100,101] However, the relevance of the ganglioside lipid composition has received minor attention in most studies and its exact effects still remain unidentified. This work investigates the effect of the ganglioside lipid chain on the immunogenicity of cancer vaccines as well as new methodologies to enhance the response to these constructs.

Analogues of GM_3 and GM_2 were chemoenzymatically synthesized on a multifunctional ceramide-type tether designed to facilitate glycoconjugate synthesis. The truncated ceramide aglycon maintains the stereogenic centers of natural ceramide while providing two sites for conjugation or elaboration of the lipid. Furthermore, this ceramide analogue avoids the extensive hydrophobicity characteristic of natural ceramide allowing for enzymatic elaboration of the glycan. These features permitted us to generate, from a single lactoside precursor, several ganglioside analogues with variations at the glycan and lipid chains.

These functionalized oligosaccharides were conjugated to the carrier protein tetanus toxoid and the ability of the different glycoconjugates to raise a ganglioside-specific immune response was evaluated in mice using a standard immunization protocol. We also explored, in collaboration with the group of Dr. Suresh (Pharmacy Department, University of Alberta), a novel strategy to augment the immunogenicity of ganglioside conjugates *in vivo via* targeting to dendritic cells.

The development of ganglioside-based cancer vaccines requires a large amount of ganglioside in a form that can be easily manipulated without disrupting any recognition elements. The chemoenzymatic synthesis initially used in our work was limited by the scale of the reaction that could be practically applied. As part of this work, we investigated the possibility of using metabolically engineered bacteria to produce ganglioside derivatives incorporating a truncated ceramide aglycon. Using this technology, complex gangliosides, including GM_3, GM_2, GM_1, GD_3, GD_2, and GT_{1a} were produced very efficiently on a large scale.

Chapter 2
Chemoenzymatic Synthesis of Ganglioside Analogues

2.1 Chemistry of Sphingosine and Gangliosides

Ganglioside analogues containing functional groups that facilitate flexible conjugation strategies constitute a valuable tool for the design and development of cancer vaccines as well as for the study and modulation of ganglioside functions at membrane interfaces. This chapter describes a chemoenzymatic synthesis of versatile GM_3 and GM_2 ganglioside analogues designed for conjugation *via* their ceramide moiety. The inherent flexibility to functionalize these analogues with different linkers for covalent attachment to proteins, surfaces, or fluorescent probes is also demonstrated.

2.1.1 Previous synthesis of sphingosine

Glycosphingolipids are important structural and functional constituents of the plasma membrane of essentially all eukaryotic cells. They have been shown to play a crucial role in many physiological processes including cell recognition,[102] adhesion,[103] growth,[18,104,105] differentiation,[105,106] signaling,[18,102,103,107,108] and apoptosis.[109-111] Each glycosphingolipid is composed of a hydrophobic ceramide core attached to a hydrophilic oligosaccharide head group which protrudes from the membrane surface. The ceramide is, in turn, composed of a long chain amino alcohol, the sphingosine base acylated with a fatty acid (Figure 2.1). Glycosphingolipids greatly differ in the structure of their carbohydrate head group and in the length, unsaturation and hydroxylation of their sphingosine base. Accordingly, they are classified into different families depending on variations in their carbohydrate head group.[112] Cerebrosides contain a single sugar residue, sulfatides contain a sulfated sugar residue, neutral glycosphingolipids contain oligosaccharides with up to 30 neutral residues, and gangliosides contain sialylated oligosaccharides.

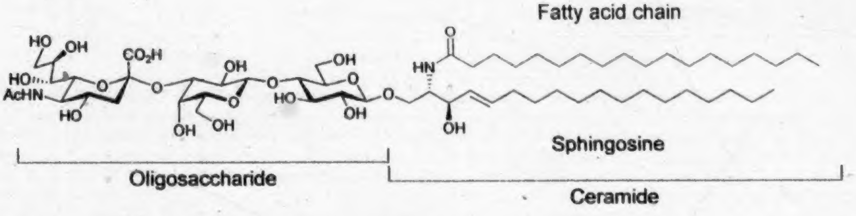


Figure 2.1 *Structural features of glycosphingolipids.*

Given the biological significance of glycosphingolipids, much effort have been invested in the synthetic preparation of these compounds and related derivatives. A key to their successful preparation lies in the availability of appropriate sphingosine bases, the principle backbone of glycosphingolipids. Although a number of related structures are known, the most frequently occurring sphingoid base in nature is D-*erythro*-2-amino-1,3-octadec-4*E*-ene-diol, commonly refered to as sphingosine (Figure 2.2). Over the past 50 years, many synthetic methodologies have been developed to access this important compound in enantiomerically pure form. The synthesis of sphingosine has been accomplished *via* two main approaches: 'the chiral pool approach' and the 'asymmetric induction approach' (Figure 2.2). In the 'chiral pool approach', the synthesis takes advantage of the availability of naturally occurring and inexpensive chiral starting materials such as amino acids and sugars. These synthetic procedures typically involve the use of the chiral material as a template to construct the 2-amino-1,3-diol head of the sphingosine either directly or through its influence on another chiral centre. An aliphatic chain is then attached to the sphingosine head group in various ways such as olefin cross-metathesis,[113] Wittig reaction,[114] or Julia olefination.[115] This approach is particularly beneficial for large-scale preparation of diastereomerically pure sphingosine. On the other hand, the 'asymmetric induction approach' starts with achiral material. The absolute stereochemistry of sphingosine is installed *de novo* using methods such as Sharpless asymmetric epoxidation[116] and asymmetric aldol condensation.[117] Contrary to the 'chiral pool approach', these methods are particularly valuable as each diastereoisomer of sphingosine is equally accessible.

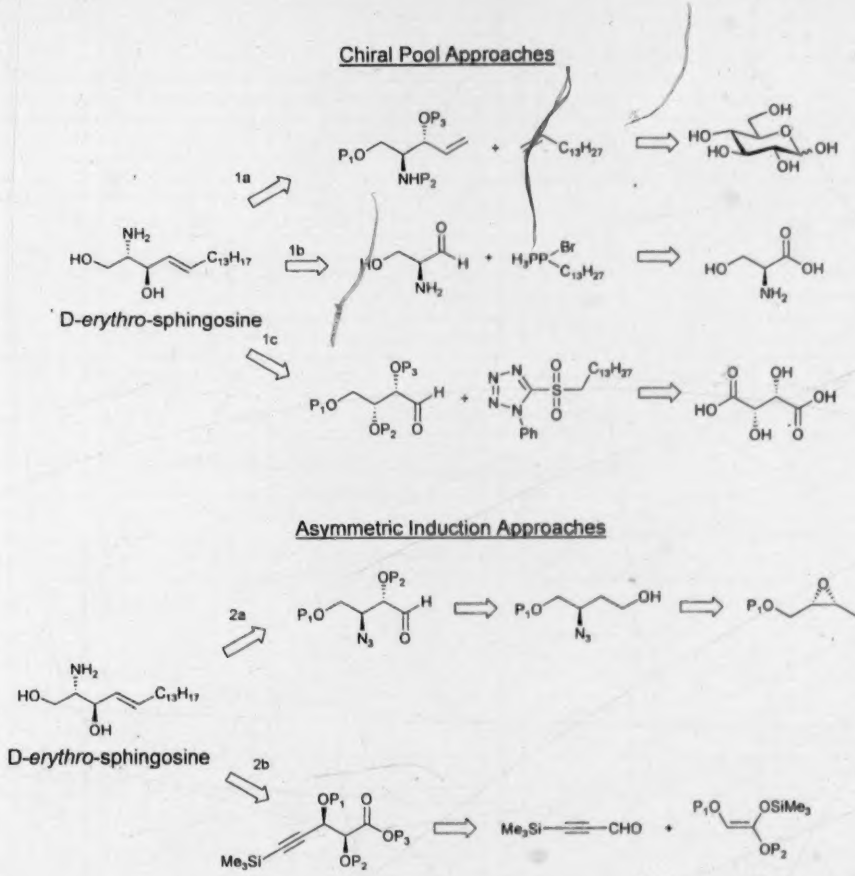
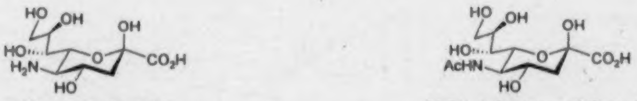

Chiral Pool Approaches
1a
1b
1c
D-erythro-sphingosine
Asymmetric Induction Approaches
2a
2b
D-erythro-sphingosine

Figure 2.2 *Strategies for the synthesis of sphingosine 1a Olefin cross-metathesis between a protected truncated sphingosine derived from glucose and 1-pentadecene. 1b Wittig coupling of an aldehyde and tetradecyltriphenylphosphonium bromide. 1c. A protected derivative of* D*-tartrate is treated under the Kocienski modification of the Julia olefination protocol. 2a. Sharpless asymmetric epoxidation is used to construct a truncated aldehyde derivative which can be further extended using the Wittig coupling or Julia olefination. 2b. Asymmetric aldol condensation yield a shorten sphingosine derivative which can be further elaborated to the full lipid.*

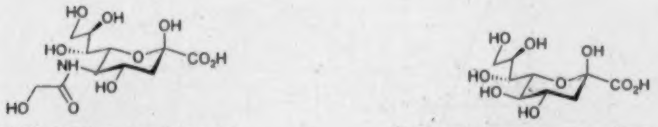
2.1.2 Previous chemical synthesis of gangliosides

Gangliosides are distinguished from other glycosphingolipids by the presence of a sialic acid moiety. As with other glycosphingolipids, gangliosides have been shown to participate in vital processes of the immune and nervous systems *via* cell-cell and cell-ligand interactions. Being exposed at the terminal end of gangliosides, the sialic acid residues are central to mediate these interactions by masking certain oligosaccharide epitopes or by acting as recognition elements for receptors and pathogens.[118] Sialic acids consist of a family of over thirty derivatives of the 9 carbon ketose neuraminic acid moiety (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid) that are generally *N*-acylated to form either *N*-acetyl or *N*-glycolyl neuraminic acid (Figure 2.3). Due to its predominance, *N*-acetyl neuraminic acid is frequently simply referred to as sialic acid. This sugar residue is an integral component of glycolipids, glycoproteins and polysaccharides where it is found as a terminal residue attached to either galactose or *N*-acetyl galactosamine *via* an α-(2→3) or an α-(2→6) linkage. Sialic acid residues can also be attached to one another *via* an α-(2→8) or an α-(2→9) linkage (Figure 2.4).[119] The structural features of sialic acid, namely its quaternary anomeric centre and its 3-deoxy-ulosonic acid motif, make difficult the introduction of sialic acid residues in oligosaccharides and have been a challenge to overcome in the synthesis of gangliosides.[119]



Neuraminic acid
(Neu)

N-Acetyl neuraminic acid
(Neu5Ac)

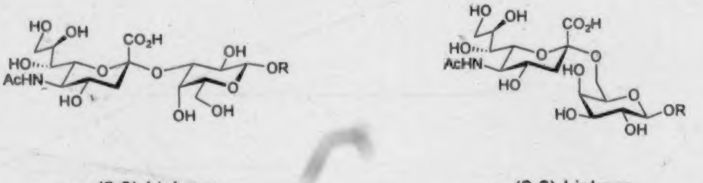


N-Glycolylneuraminic acid
(Neu5Gc)

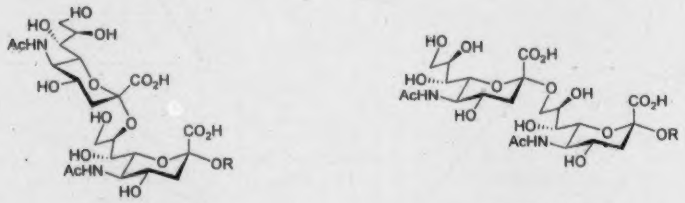
3-Deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid (KDN)

Figure 2.3 *Structure of common sialic acids.*

The synthesis of gangliosides can be divided into three steps: (1) the construction of the oligosaccharide backbone, (2) the introduction of the sialic acid residue(s) into the oligosaccharide backbone, and (3) the coupling of the sialylated oligosaccharide to the ceramide aglycon. Two important directing lines have been used in the chemical synthesis of gangliosides. First, the oligosaccharide should be completely built prior to coupling to the lipid portion as the reactivity of the resulting glycolipid is too low to allow for elaboration of the oligosaccharide moiety in good yield. Furthermore, owing to the low reactivity and succeptibility of sialosyl donors to 2,3-elimination, sialic acid should be introduced at an early stage of the oligosaccharide synthesis and preferably coupled with a reactive acceptor.[120]


α-(2,3)-Linkage

α-(2,6)-Linkage


α-(2,8)-Linkage

α-(2,9)-Linkage

Figure 2.4 *Common sialosyl linkages.*

The synthesis of gangliosides presents several important difficulties that have limited access to this important class of glycolipid (Figure 2.5).[119] First, the introduction of sialic acid into the oligosaccharide backbone remains problematic both in terms of yield and stereoselectivity. The reactivity of sialosyl donors is particularly low as glycosylation occurs at a sterically hindered tertiary oxocarbenium ion intermediate. Furthermore, the deoxy moiety in combination with the electron-withdrawing carboxylic acid at the anomeric centre render sialosyl donors particularly susceptible to 2,3-elimination to form the corresponding glycal, further decreasing the glycosylation yield (Figure 2.5A). The development of synthetic strategies to access sialosides have focused on the formation of the natural α-anomer. However, this stereoisomer is difficult to obtain as the opposite configuration, the β-anomer, is thermodynamically favored and neighboring group participation cannot be used to direct the stereochemical outcome of the coupling reaction

(Figure 2.5B). These challenges have been addressed by a pleiotropy of direct and indirect methods that will be surveyed in the following sections.

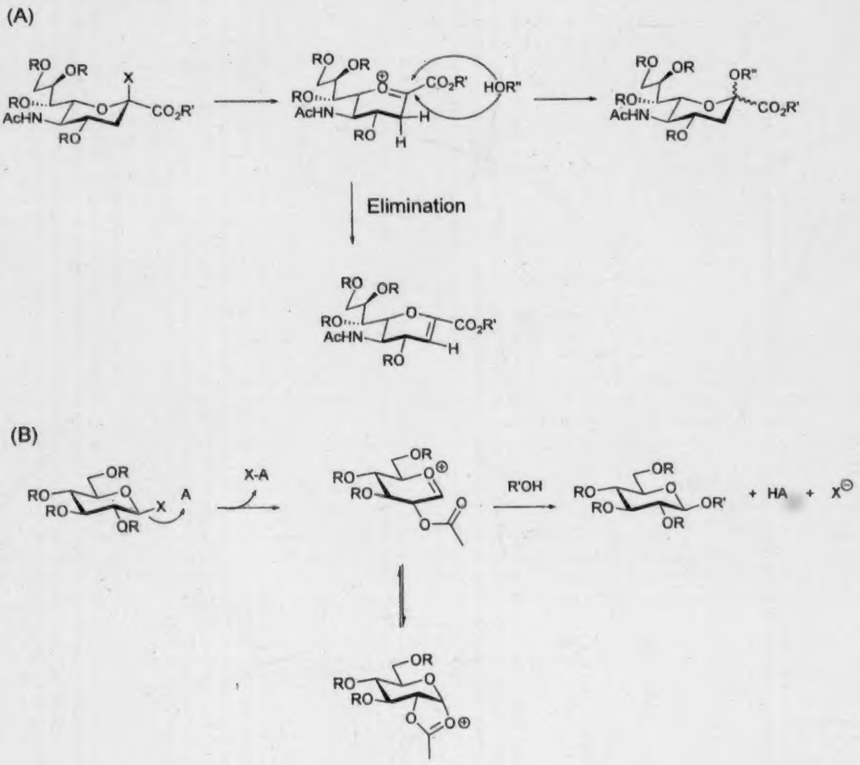
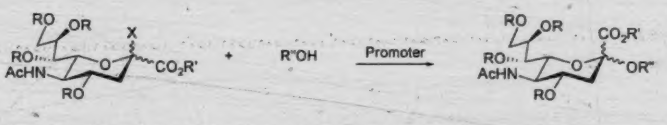


Figure 2.5 *Problems associated with chemical sialylation. (a) Formation of sialosyl glycal via 2,3-elimination. (b) Example of the use of neighboring group participation in directing the stereochemistry of a glycosylation reaction.*

Early methods for the formation of sialosides involved the use of 2-chloro derivatives of sialic acid under Koenings-Knorr or Helferich conditions (Figure 2.6).[121] Owing to their ease of synthesis and their stability, sialyl chlorides were considered ideal donors compared to other 2-halogeno derivatives. However, these methods afford the α-ketosides in modest yields and are limited to glycosylation with simple alcohols.[122] More modern glycosylation methodologies such as 2-thio derivatives,[29,119,123,124] 2-trichloroacetimidates, 2-xanthates,[123] and 2-phosphites[125, 126] have also been applied with some success for the formation of α-sialosyl linkages. However, the use of these donors were not enough to significantly improve the yield and stereoselectivity of the glycosylation. Thus, other strategies needed to be devised.



X = Br, Cl, F, SMe, SEt, SPh, SC(=S)OEt, OP(OR")$_2$, OCOR'

Figure 2.6 *Direct chemical methods for the formation of sialosyl linkages.*

Thioglycosides are one of the most important class of donors in carbohydrate chemistry due to their stability, versatility, and compatibility with many reaction conditions.[29] These donors have also been applied, with some success, to the synthesis of sialyl-containing oligosaccharides. The best results were obtained when a participating solvent was used at low temperature to direct the α-selectivity of the reaction (Figure 2.7).[127, 128] The reaction of the thioglycoside with DMTST in acetonitrile involves the generation of an electrophilic species ($^+$SMe) which reacts with the lone pair of the sulfur atom to give a sulfonium intermediate. The sulfonium intermediate is an excellent leaving group and can be displaced by the nitrogen atom of acetonitrile, resulting in the formation of a nitrilium ion which preferentially adopts the β-configuration. Subsequent nucleophilic displacement of the nitrilium ion by the hydroxyl group of a glycosyl donor gives predominantly an α-glycoside.[128, 129] Further improvements were achieved with the use of partially protected acceptors[130-132] and highly reactive promoters such as NIS/ TfOH.[128] Activation of thioglycosides requires at least an equimolar amount of a thiophilic promoter. This major drawback was addressed by the use of phosphite donors. Phosphite donors are very reactive and require only a catalytic amount of TMSOTf for initiation of glycosylation.[125,126] The stereochemistry at the anomeric position is favorably set due to the preferred β-orientation of the phosphite group which insures predominant formation of the α-product.[133] Application of this method gives the desired α-sialoside product in yields and anomeric selectivity comparable to those obtained with thioglycoside donors.

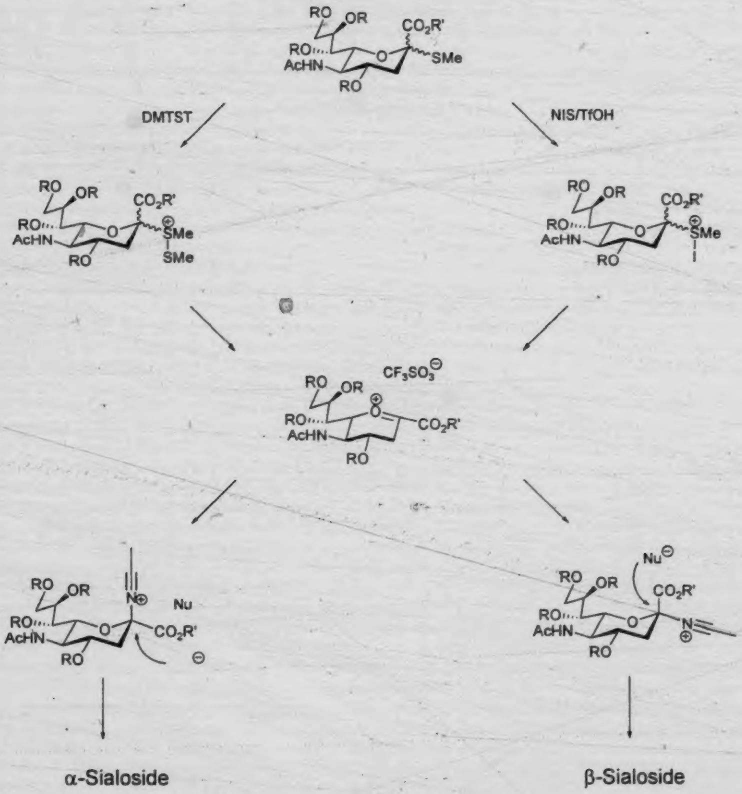


Figure 2.7 *Directing α-product using solvent participation in sialylation reactions.*

Apart from using different types of glycosyl donors, problems in sialoside synthesis have been addressed by introducing an auxiliary to obtain a modified sialyl donor harboring a participating group (Figure 2.8). Different auxiliaries including 2-thioethyl ester, 3-*O*,[134-136] 3-bromo-,[137] 3-thio-,[138,139] and 3-seleno-[140] derivatives have been reported. These auxiliaries control the anomeric selectivity by providing a participating group. These approaches can be divided into two groups depending on the position of the auxiliary. The carboxyl group at *C-1* of 2-thioethylmethyl neuraminic acid donor can be protected as a 2-thioethyl ester. Activation of the anomeric centre of the donor would yield an oxonium-ion intermediate which is stabilized by long-range participation of the thiomethyl moiety resulting in the formation of a sulfonium intermediate. Glycosylation of the thermodynamically favored β-sulfonium ion affords mainly the α-sialoside (Figure 2.8A).[141] Several sialosyl donors containing an auxiliary at the *C-3* position have been used in the synthesis of sialosides.[119,133] An equatorial auxiliary at *C-3* provides a group for neighboring participation leading to the formation of a 2,3-*trans* linkage to yield the corresponding α-sialoside. In addition, the auxiliary at *C-3* stabilizes the oxocarbenium ion intermediate thus preventing the formation of glycal as a side product. Despite the many developments in chemical sialylation, there exists no general method that can be applied with success to a wide range of glycosyl acceptors. In recent years, the problems associated with the installation of sialic acid moieties have been addressed by enzymatic methods. Enzymatic sialylations have proved to be a powerful way to stereoselectively prepare sialosides in high yield without the need of cumbersome protecting group manipulations.[48, 142-147] These methods will be discussed in Section 2.2.

Typical reaction schemes to build glycosphingolipids involve the coupling of either the complete ceramide or a truncated version of the lipid (Figure 2.9) to the oligosaccharide moiety. A protecting group which can participate in anchimeric assistance is installed at the C-2 position of a glucopyranose derivative to direct the formation of the β-glycoside. Although the coupling of the complete ceramide (Route 1 in Figure 2.9) offers a shorter synthetic route, the yield of the reaction is generally low as the reactivity of the primary alcohol in the ceramide (and sphingosine) is diminished by hydrogen bond formation with the amide proton (Figure 2.10A).[148] Azidosphingosine derivatives (Route 2 in Figure 2.9) have proven to be superior acceptors due to their enhanced reactivity and are now preferably employed.[149] However, the low solubility in organic solvent and the propensity of full-length sphingosine derivatives to form aggregates often result in low reaction yields and purification problems.[150] These problems have been addressed by the use of a truncated azidosphingosine analogue (Route 3 in Figure 2.9).[151-153] The increased reactivity of the lipid acceptor coupled with better solubility properties have made these derivatives the acceptor of choice in the construction of glycosphingolipids. Although the resulting glycolipid requires further transformation, these donors consistently afford good coupling yields while offering the flexibility to derivatize the glycosphingolipid with different fatty acid chains.

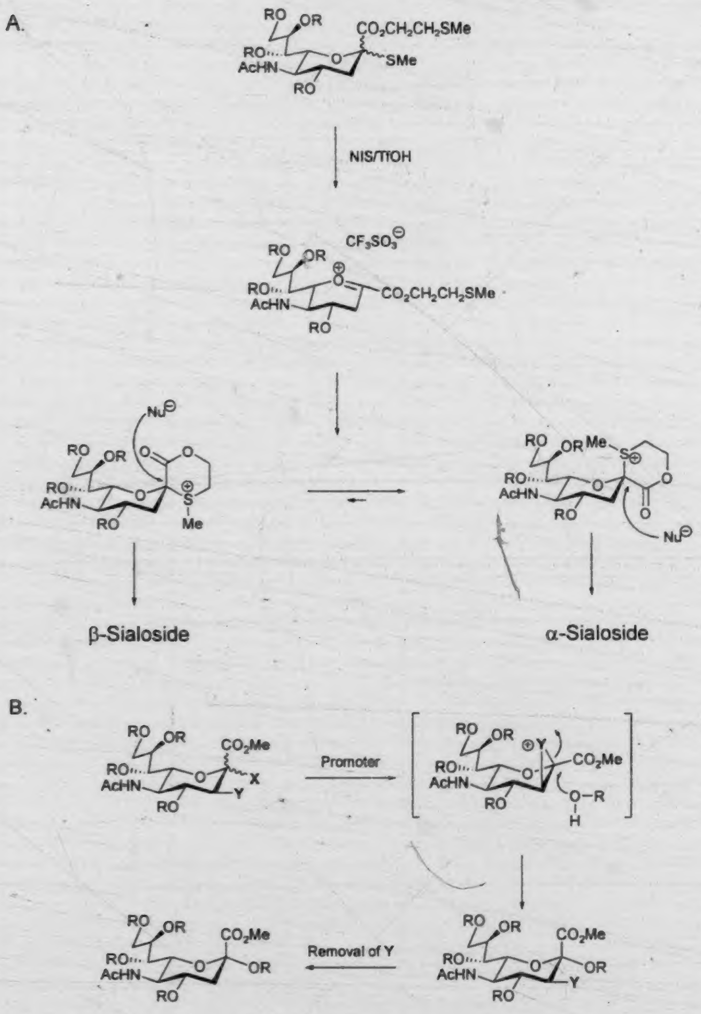

A.
CO2CH2CH2SMe
SMe
NIS/TfOH
CF3SO3⊖
CO2CH2CH2SMe
Nu⊖
β-Sialoside
α-Sialoside
B.
CO2Me
X
Y
Promoter
Removal of Y
RO
OR
AcHN

Figure 2.8 *Directing α-sialoside selectivity using auxiliaries. (a) Long-range participation of a C-1 auxiliary. (b) Neighboring participation of a C-3 auxiliary.*

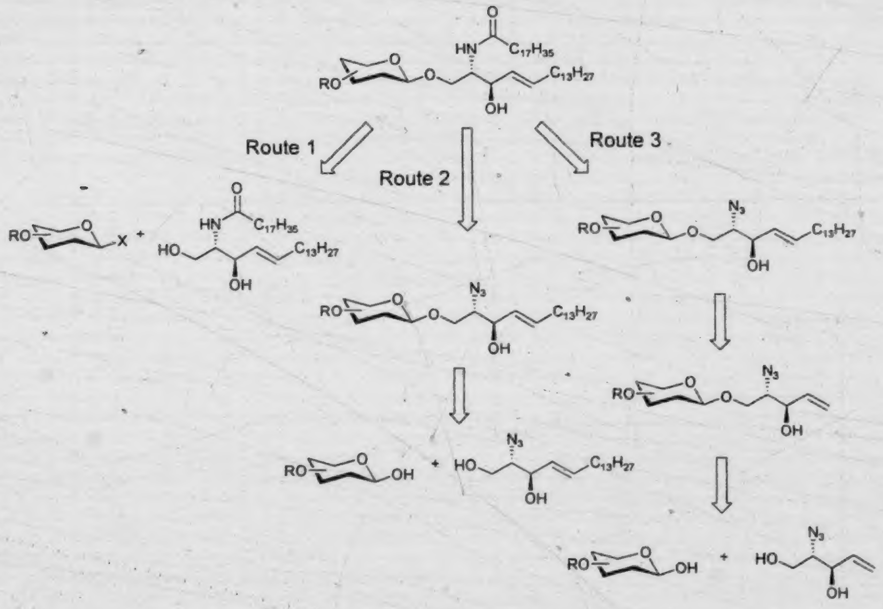

Route 1
Route 2
Route 3
RO
O
X
HN
HO
OH
$C_{17}H_{35}$
$C_{13}H_{27}$
N_3

Figure 2.9 *Strategies for the synthesis of glycosphingolipids.*

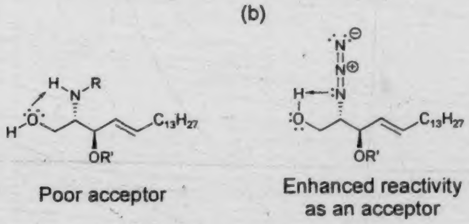

(a)
(b)
Poor acceptor
Enhanced reactivity
as an acceptor
$C_{13}H_{27}$
OR'

Figure 2.10 *Interactions (a) disfavoring / (b) favoring glycosylations with sphingosine derivatives.*

2.2 Enzymatic methods for the synthesis of gangliosides

Due to their utility, most enzymatic synthesis of gangliosides involve an enzymatic sialylation step. These methods typically involve the partial synthesis of an oligosaccharide fragment followed by the use of an enzyme to establish one or more glycosidic linkages. Due to the hindered tertiary anomeric centre and the absence of neighboring group participation, chemical sialylation is considered one of the most difficult glycosylations in carbohydrate chemistry.[119] Enzymatic methods have proved very useful in the synthesis of sialylated structures, avoiding many of the problems associated with chemical approaches including extensive protection/deprotection schemes and formation of numerous diastereoisomeric products. Two fundamentally different approaches have been developed for the enzymatic preparation of sialosides: the use of sialyltransferases and the application of sialosidases.

2.2.1 Glycosyltransferases in the synthesis of gangliosides

Sialyltransferases transfer with high regio- and stereoselectivity a sialic acid residue from an activated cytidine 5'-monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) donor to the hydroxyl group of an appropriate acceptor. Several types of sialyltransferases have been used in the synthesis of sialosides. α-2,6-Sialyltransferases[154-160] and α-2,3-sialyltransferases[56,159,161-167] have been used to transfer a sialic acid moiety to either the C-6 or C-3 hydroxyl group of galactosyl- and *N*-acetyl galactosaminyl residues, whereas α-2,8- and α-2,9-sialyltransferases[167] have been utilized to construct polysialic acid fragments. The requirement of an expensive nucleotide sugar donor can limit the enzymatic production of large quantities of sialosides. This limitation has been overcome *via* numerous strategies, the most effective of which involves the generation of CMP-Neu5Ac *via* a CMP-Neu5Ac synthase–catalyzed condensation of neuraminic acid with CTP (Figure 2.11).[168-172] Furthermore, an *in situ* cofactor regeneration system for CMP-Neu5Ac can be devised in which the CMP formed after the silylation is recycled *via* a series of enzymatic reactions (Figure 2 .11), making this one-pot process even more efficient.[171, 173]

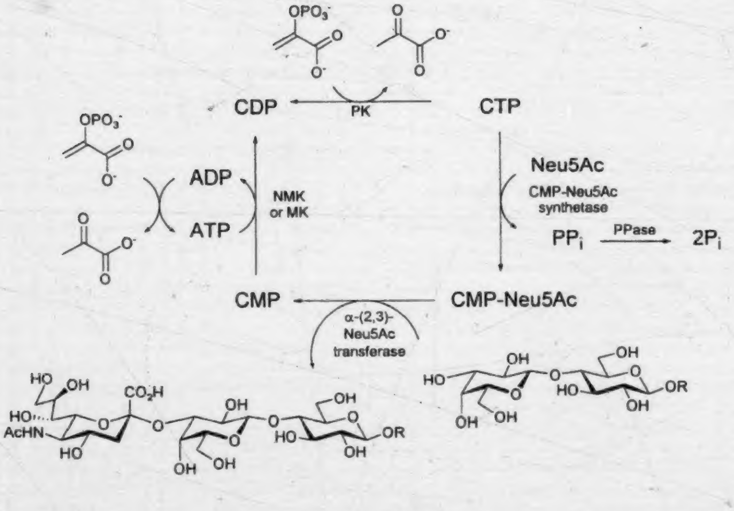


Figure 2.11 *Enzymatic sialylation coupled with sugar nucleotide donor regeneration.*

2.2.2 Glycosidases in the synthesis of gangliosides

Enzymatic sialylation using sialidases have also been investigated.[174,175] The relaxed substrate specificity of these enzymes compared to glycosyltransferases is advantageous in the preparation of sialosides. Sialidases are glycosyl hydrolases that generally catalyze cleavage of terminal sialic acid residues α-linked to glycoproteins, glycolipids and polysaccharides. As the reaction catalyzed by these enzymes is reversible, sialylation can be effected under conditions favoring the reverse *trans*-glycosylation. Depending on the origin of the sialidase, different regioselectivity can be obtained. A *trans*-sialidase from *tropanosoma cruzi*[176-179] have been widely employed to form α-(2,3)-linked sialosides whereas sialidases from *vibrio cholerae* have been used to form α-(2,6)-linked sialosides.[175,180] Both enzymes display a very low glycosyl transferring activity and can afford sialosides in low yields. However, through the rational engineering of the enzyme active site, it is possible to obtain a 'glycosynthase' whose hydrolase activity is greatly reduced without affecting the transferase activity.[58,59,181,182] These glycosynthases are mutants retaining glycosidases in which the catalytic nucleophile is replaced with an inactive residue. When presented with a glycosyl fluoride of the opposite anomeric configuration which mimics the glycosyl enzyme intermediate, the mutant enzyme can catalyse its glycosylation with another sugar residue. This modified enzyme produces the desired oligosaccharide in significantly higher yield. Unfortunately, such an enzyme construct is not yet available for the preparation of sialosides.

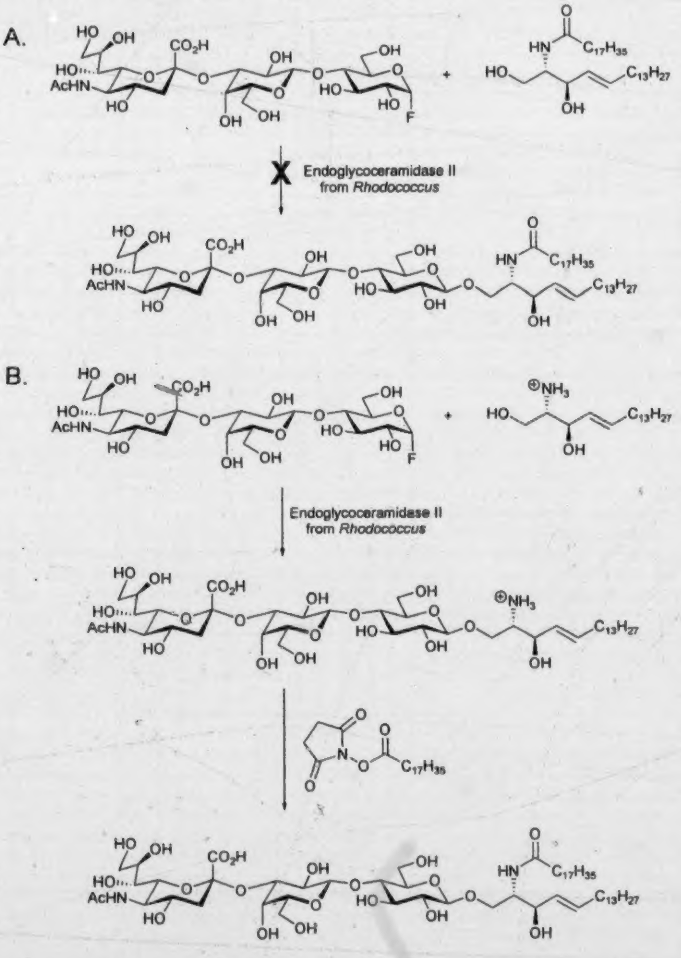

A.
Endoglycoceramidase II
from *Rhodococcus*
B.
Endoglycoceramidase II
from *Rhodococcus*

Figure 2.12 *Glycosynthase-mediated synthesis of glycosphingolipids.(a) Synthesis of GM$_3$ using N-palmitoyl-D-erythro-sphingosine as an acceptor. (b) Synthesis of GM$_3$ using D-erythro-sphingosine as an acceptor followed by subsequent acylation with N-palmitoyloxysuccinimide.*

Efforts in the enzymatic synthesis of gangliosides are not limited to the formation of the sialosyl portion of the molecule. Recently, novel developments were made for the formation of the synthetically challenging bond between an oligosaccharide and sphingosine moiety (Figure 2.12). The enzymatic coupling of the sphingosine lipid to a preassembled oligosaccharide was achieved by a glycosynthase engineered from the well-studied endoglycoceramidase II (EGC II). For example, when 3'-sialyllactosyl fluoride and D-*erythro*-sphingosine were incubated in the presence of the glycosynthase, the target glycolipid was obtained in high yield (>95%).[183] Although the *N*-palmitoyl-D-*erythro*-sphingosine cannot act as an acceptor due to its very low aqueous solubility, the natural compound can be easily obtained by acylation of the *lyso* analogue using an activated palmitoyl ester derivative. This method, in combination with enzymatic glycosylations, holds great promise for the efficient and affordable preparation of gangliosides.

2.3 Target structures

Gangliosides are highly overexpressed in melanoma and other tumors of neuroectodermal origin and thus constitute attractive targets for the development of tumor immunotherapy.[77] Vaccines based on ganglioside-KLH conjugates have shown promising results in the clinic.[86,88,184-186] However, in order to uphold their promise as a therapeutic agent, the immunogenicity of these glycoconjugates should be greatly enhanced. The scarce availability of the glycolipid in a form amendable to conjugation poses a major limitation to the development of these vaccines. In this context, a methodology designed to readily access ganglioside analogues functionalized for conjugation is particularly valuable. Several lines of evidence in the literature relating to ganglioside recognition by proteins implicate the ceramide portion of the glycolipid either directly or *via* its influence on the conformation of the oligosaccharide. It is thus hypothesized that not only the oligosaccharide portion, but also the ceramide moiety of gangliosides, are important for their functions. However, the ampiphilic nature of gangliosides greatly complicates their synthesis. Due to their hydrophobic lipid chain and their hydrophilic head group, gangliosides exhibit surfactant-like properties such as low solubility in organic solvents and aqueous solutions as well as aggregation resulting in low reaction yields and purification problems.[150] In order to avoid these problems and include a tandem recognition of elements of the oligosaccharide and ceramide moiety, a truncated version of sphingosine **1** was used to build the ganglioside analogues reported here. This truncated sphingosine aglycon incorporates the essential stereochemical features of naturally occurring ceramide and functional groups facilitating coupling of the glycolipid to different carriers. In addition to the flexibility afforded to the conjugation strategy by the presence of the terminal alkene and the latent amino group, an additional advantage of this aglycon is conferred by the well established superiority of azidosphingosines over ceramides as glycosyl acceptors. Following glycosylation, the azidosphingosine can be derivatived either by addition of a thiol such as cysteamine to the alkene or by sequential azide reduction and *N*-acylation. Furthermore, the truncated azidosphingosine derivatives exhibit increased aqueous solubility when compared to their ceramide counterparts allowing enzymatic elaboration of the oligosaccharide portion to complex sialylated structures while avoiding long and tedious chemical transformations. To demonstrate the potential of our methodology, two of the simplest gangliosides, a GM_2 analogue **2** and a GM_3 analogue **3**, were selected as targets (Figure 2.13).

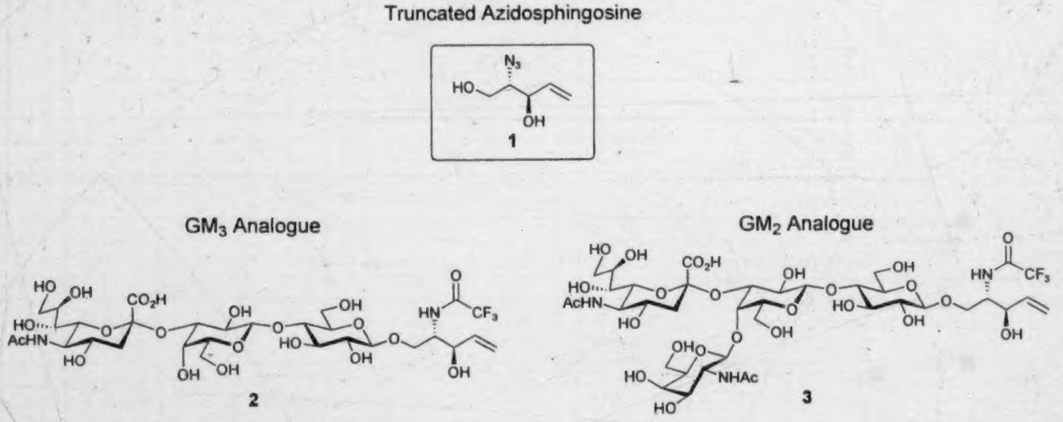

Truncated Azidosphingosine
N3
HO
OH
1
GM3 Analogue
HO
OH
CO2H
HO
AcHN
HO
O
O
OH
O
OH
OH
OH
O
HO
OH
O
O
HN
O
CF3
OH
2
GM2 Analogue
HO
OH
CO2H
HO
AcHN
HO
O
O
OH
O
O
OH
OH
O
HO
OH
O
O
HN
O
CF3
OH
OH
HO
HO
HO
NHAc
3

Figure 2.13 *Target Structures.*

2.4 Results and Discussion

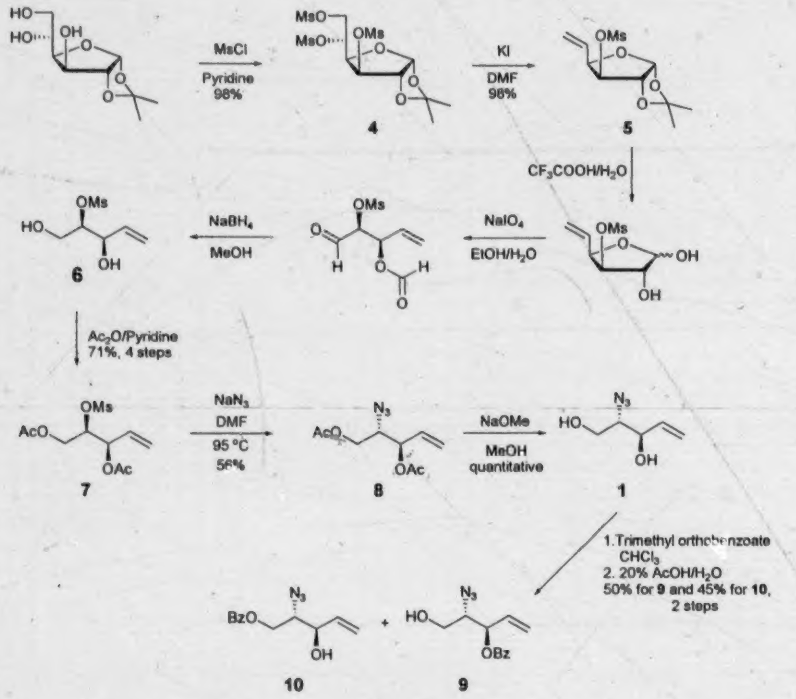
2.4.1 Synthesis of truncated lactosyl sphingosine analogue 11

Azidosphingosine analogue **1** was synthesized according to a procedure developed in our group by Dr. Chang-Chun Ling.[153] The transformation sequence started with permesylation of commercially available 1,2-*O*-isopropylidene-α-D-glucofuranose (Scheme 2.1). Unlike standard sulfonations, which require vigorously anhydrous conditions, treatment of 1,2-*O*-isopropylidene-α-D-glucofuranose with 4.0 equivalents of methanesulfonyl chloride in reagent grade pyridine afforded complete mesylation. Trimesylate **4** was isolated in almost quantitative yield by simple precipitation in ice-cold water. Literature procedures for iodine-mediated deoxygenation at the 5,6-position of furanose rings typically involve extended reflux in 2-butanone in the presence of excess sodium iodide. However, in this case, these conditions lead to incomplete deoxygenation. It was subseqently found that treatment of **4** with potassium iodide in *N,N*-dimethylformamide at 90°C resulted in a faster and complete reaction. Compound **5** was obtained in essentially pure form by extraction with ethyl acetate and an aqueous solution of sodium thiosulfate. Transformation of **5** to **7** involves a four-step sequence. First, the 1,2-*O*-isopropylidene ring of **5** was opened in a solution of 50% aqueous trifluoroacetic acid under gentle heating (55 °C). After 2 hours, thin layer chromatography indicated quantitative deprotection. The hemiacetal obtained was then oxidized with sodium periodate in water under slightly basic conditions (pH 8.5) to prevent formation of any Fisher glycosylation side product. These conditions yielded an intermediate aldehyde and its hydrate which were isolated by extraction. The aldehyde functionalities were then reduced with sodium borohydride in methanol to afford a diol. Subsequent nucleophilic displacement of the mesylate by sodium azide was problematic and afforded the target product **7** in poor yield. However, protection of the hydroxyl groups with acetates prior to the displacement reaction gave improved results. Treatment of **7** with excess sodium azide in DMF at 95 °C afforded the target compound **8** in moderate yield. Formation of several side products justified the need of a chromatographic purification at this stage.

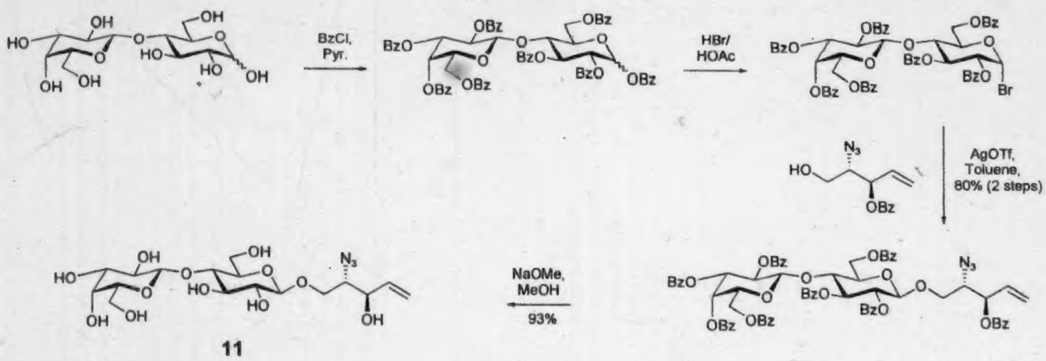
Zemplén transesterification then cleanly removed the acetates of **8** to give diol **1** in quantitative yield.

In order to couple a carbohydrate moiety to truncated azidosphingosine **1**, its secondary hydroxyl group must be selectively protected. We envisaged the use of a protecting group strategy that would allow for the deprotection of the final lactoside in a single step. Benzoyl groups have been used extensively in carbohydrate chemistry to block hydroxyl groups and direct formation of the β-glycosidic bond. We thus elected this protecting group for building our β-lactoside. It is well known that primary alcohols can be selectively protected using a sterically hindered group such as trityl or *tert*-butyldimethylsilyl. A reaction sequence in which the primary alcohol was first selectively protected, followed by benzoylation of the secondary alcohol and re-opening of the primary position could be conceived. However, this approach involves a cumbersome three-step reaction sequence. We thus rely on the formation of an orthobenzoate ester to directly introduce the benzoyl group on the secondary alcohol. This reaction worked very well by following a rapid one-pot, two-stage reaction sequence. Diol **1** was first treated with trimethyl orthobenzoate in the presence of a catalytic amount of camphorsulfonic acid in chloroform. After complete disappearance of the starting material, the cyclic orthoester was opened with a 20% aqueous acetic acid solution to afford a 1:1 mixture of **9** and **10**. The overall yield of the transformation was excellent and the two isomers were readily separated by column chromatography on silica gel. To avoid any waste of reaction intermediate, the undesired isomer **10** can be quantitatively recycled by a simple Zemplén transesterification.

The selectively protected truncated azidosphingosine **9** was then glycosylated with a lactosyl derivative (Scheme 2.2). After studying several different donors (thioglycosides, imidate), it was found that lactosyl bromide gave the best results. Initially, a peracetylated lactosyl donor was used in the glycosylation reaction to provide a participating neighboring group to favor the formation of a β-linkage. However, the orthoester intermediate fails to completely rearrange, even in the presence of a base, to form the desired glycoside. We thus changed, with success, our participating neighbouring group to a benzoyl group, which forms a less stable orthoester intermediate. Glycosidation of perbenzoylated lactosyl bromide donor with the truncated azidosphingosine **1** using silver triflate as a promoter afforded the corresponding crude lactoside in high yield. The intermediate glycoside was not purified but rather subjected directly to Zemplén transesterification conditions to give the target lactoside **11** in excellent yield after reverse-phase purification.


MsCl
Pyridine
98%
4
KI
DMF
98%
5
CF₃COOH/H₂O
NaIO₄
EtOH/H₂O
NaBH₄
MeOH
6
Ac₂O/Pyridine
71%, 4 steps
7
NaN₃
DMF
95 °C
56%
8
NaOMe
MeOH
quantitative
1
1.Trimethyl orthobenzoate
CHCl₃
2. 20% AcOH/H₂O
50% for 9 and 45% for 10,
2 steps
10
9

Scheme 2.1 *Synthesis of truncated azidosphingosine* **1** *and its selectively protected derivative* **9**.



Scheme 2.2 *Synthesis of truncated lactosyl sphingosine* **11**.

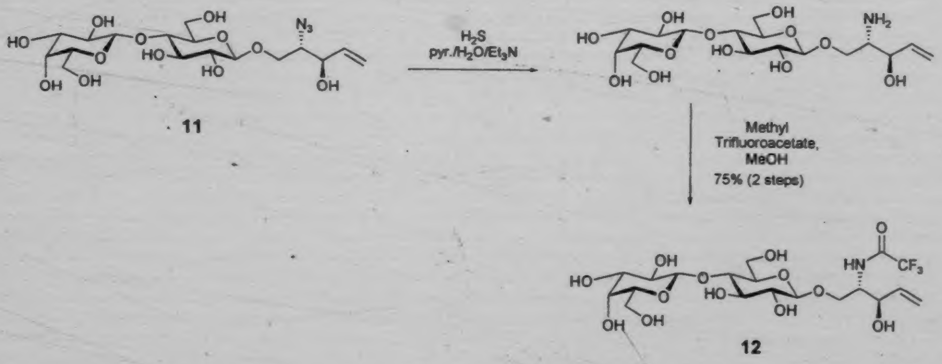
2.4.2 Chemoenzymatic synthesis of GM_3 and GM_2 ganglioside analogues[1]

Our chemoenzymatic approach was centered on a versatile lactosyl ceramide analogue **12**, which could be elaborated to gangliosides of higher complexity or functionalized with different acyl chains at the sphingosine amine. Intermediate **12** was synthesized as outlined in Scheme 2.3. The azide in compound **11** was first reduced to afford the amine. Application of common reduction methods such as Staudinger conditions failed to afford the target compound, presumably due to the formation of a aziridine intermediate. An alternate strategy was thus employed in which the azide was reduced in the presence of hydrogen sulfide in a solution of pyridine, water, and triethylamine to afford the amine in high yield. The use of the free amine in enzymatic reactions has proven to be impractical in our hands. Therefore, we envisioned that protection of the amine group would ease purification of the compound in subsequent enzymatic reactions. Due to the acid lability of the *N*-acetyl neuraminic acid residue, an amine protecting group which could be removed under mildly basic conditions, was preferred. Trifluoroacetamide was selected as the amino protecting group as it can be easily installed and leads to the formation of a volatile by-product upon deprotection. Thus, the free amine was acylated by reaction with methyltrifluoroacetate to afford **12** in 75% yield. The moderate yield of the protection can be explained by the partial formation of a trifluoroacetate salt which greatly reduces the reactivity of the amine being protected. The salt could be isolated from the reaction mixture and the free amine regenerated by elution though a OH$^-$ ion-exchange column.

---

[1] Molecular biology work in this section was carried out by the group of Dr. Warren Wakarchuk at the National Research Council of Canada (NRC). The author would like to thank Dr. Wakarchuk for kindly providing different bacterial strains expressing glycosyltransferases used in this work.

Enzymatic synthesis of oligosaccharides obviates many of the problems associated with chemical synthesis, allowing access to complex compounds with high regio- and stereoselectivity in a smaller number of steps. We thus envisioned elaborating our ganglioside analogues from a truncated lactosyl-sphingosine core using glycosyltransferase-catalyzed reactions. A major drawback of glycosylation reactions catalyzed by glycosyltransferase is the requirement for expensive sugar nucleotide donors. Thus we decided to generate the sugar nucleotide donors required for our synthesis from less expensive precursors using recombinant enzymes.



Scheme 2.3 *Synthesis of lactosyl sphingosine* **12**.

2.4.2.1 Preparation of cytosine-5'-monophosphate *N*-acetyl neuraminic acid (CMP-Neu5Ac)

Cytosine-5'-monophosphate *N*-acetyl neuraminic acid (CMP-Neu5Ac) is the donor used by sialyltransferases to attach sialic acid residues to the acceptor hydroxyl group. This expensive and unstable sugar nucleotide is required in large quantities for the enzymatic synthesis of sialylated structures on a preparative scale. In this context, a CMP-Neu5Ac synthetase, an enzyme that form CMP-Neu5Ac from sialic acid and CTP, represents a valuable tool in the preparation of sialosides. We produced the enzyme from a bacterial clone (NSY-05) overexpressing a CMP-Neu5Ac synthetase from *Neisseris meningitidis*. The bacterial clone transports a pCWori$^+$ plasmid encoding the *N. meningitides* 460Y CMP-Neu5Ac synthetase gene downstream of a IPTG-inducible promoter along with an ampicillin-resistance gene as a selection marker.[187] This construct was overexpressed in *Escherichia coli* AD202 avoiding degradation of the synthetase by OmpT. The bacterial clone was grown in YT2X media containing ampicillin. Induction of the culture with IPTG (isopropyl β-D-1-thiogalactopyranoside) at log phase followed by overnight incubation afforded the enzyme in high yield (over 70 000 U/L). The enzyme was isolated by first disrupting the bacteria and recovering the crude enzyme in the supernatant after a series of centrifugations. This crude CMP-Neu5Ac synthetase preparation was used without purification in the synthesis of CMP-Neu5Ac. A CMP-Neu5Ac synthetase can prove useful in enzymatic sialylations in two different ways. In a one-pot strategy, CMP-Neu5Ac is generated from its precursors concomitant with the formation of the sialoside. Alternatively, CMP-Neu5Ac can be prepared independently and used in subsequent sialylation reactions. We preferred the latter approach as the CMP-Neu5Ac synthetase and the sialyltransferase available to us require different pH conditions for optimal activity. Overnight incubation of the crude enzyme preparation with neuraminic acid, cytosine triphosphate, magnesium chloride and dithiothreitol under optimal pH conditions effected the synthesis of CMP-Neu5Ac. Up to one gram of crude material could be obtained following sedimentation of the protein and lyophilization with an estimated conversion of 90%. This crude product was used with success in all our sialylation reactions.

2.4.2.2 Enzymatic sialylation to form a GM$_3$ analogue

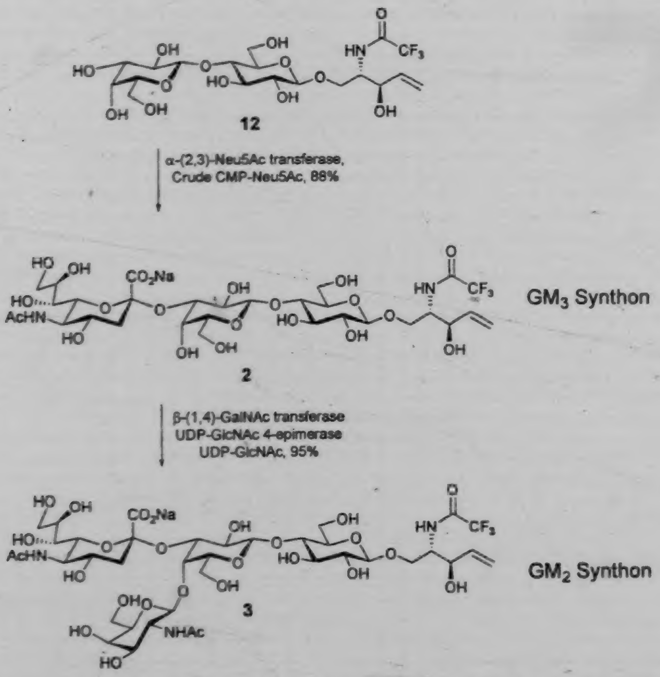
The GM$_3$ analogue **2** was generated from lactoside **12** in an enzymatic step catalyzed by an α-2,3-sialyltransferase (Cst-I) from *Campylobacter jejuni*.[188] Since the purified α-2,3-sialyltransferase is relatively insoluble and has a tendency to precipitate and lose activity upon storage, a fusion protein carrying the α-2,3-sialyltransferase along with a CMP-Neu5Ac synthetase at its *N*-terminus was used. This construct proved to be more stable and soluble than the individual proteins, while maintaining the activity of both enzymes.

Again, the fusion protein was cloned in the pCWori+ vector and expressed in an OmpT-deficient *E. coli* strain (AD202) to prevent degradation of the enzyme construct. The bacterial clone thus obtained (CST-04) was grown for 24 hrs at 25 °C in YT2X media containing 10% (v/v) glycerol. After the growth period, the enzyme was isolated by first disrupting the bacteria and recovering the crude enzyme in the pellet after a series of centrifugations. Resuspension of the pellet in the appropriate buffer afforded the crude enzyme preparation in high yield (>1200 U/L). This crude enzyme preparation was successfully used to effect sialylation avoiding significant loss of activity upon purification. Although both enzymes of the bifunctional construct were functional, we preferred to produce the CMP-Neu5Ac in a separate reaction as this method turned out to be more productive than *in situ* generation of CMP-Neu5Ac. Our methodology involves incubation of lactoside **12** with a slight excess (1.8 equivalents) of the donor CMP-Neu5Ac and the crude α-2,3-sialyltransferase preparation (Scheme 2.4).

Addition of alkaline phosphatase proved essential to cleave the inhibitory cytosine monophosphate by-product and push the reaction to completion. After this time, the reaction mixture was centrifuged to remove the magnesium pyrophosphate precipitate. The supernatant was first applied to a Sep-Pak cartridge to remove water-soluble impurities and subsequently applied to a size-exclusion column to remove small molecular weight impurities. This process rapidly afforded the GM_3 analogue **2** in good yield (88%).

2.4.2.3 Enzymatic preparation of a GM_2 analogue

The GM_3 analogue **2** was further elaborated to ganglioside GM_2 **3** using a recombinant β-(1,4)-*N*-acetylgalactosaminyl transferase (CgtA) from *C. jejuni* (Scheme 2.4). The CgtA gene was cloned in the pCWori+ vector and expressed in the host strain *E. coli* AD202.[167] Following overnight culture of the bacteria, disruption of the cells and removal of cell debris by centrifugation, the recombinant enzyme was isolated in crude form in the supernatant. This enzyme preparation was used without purification in the glycosylation reactions. Synthesis of GM_2 derivatives on a preparative-scale requires a stoichiometric amount of the expensive sugar nucleotide donor UDP-GalNAc. This motivated us to prepare the sugar nucleotide donor from a less costly precursor. A bifunctional UDP-GlcNAc/Glc 4-epimerase from *C. jejuni* was made available to us, thus allowing the synthesis of UDP-GalNAc from UDP-GlcNAc.[2] The epimerase (gne) was inserted into a pCWori+ cloning vector and expressed in *E. coli* AD202.[189] After a 24-hr culture, the cells were lysed and the crude enzyme isolated by centrifugation. GM_2 Analogues were prepared *via* a one-pot strategy in which the sugar nucleotide donor, UDP-GalNAc was generated *in situ*. GM_3 Analogue **2** was incubated overnight with UDP-GlcNAc in the presence of the β-(1,4)-*N*-acetylgalactosaminyl transferase and UDP-GlcNAc/Glc 4-epimerase enzyme preparations. Isolation of the product by solid phase extraction and size exclusion afforded the target **3** in excellent yield (95%).


12
α-(2,3)-Neu5Ac transferase,
Crude CMP-Neu5Ac, 88%
2
GM₃ Synthon
β-(1,4)-GalNAc transferase
UDP-GlcNAc 4-epimerase
UDP-GlcNAc, 95%
3
GM₂ Synthon

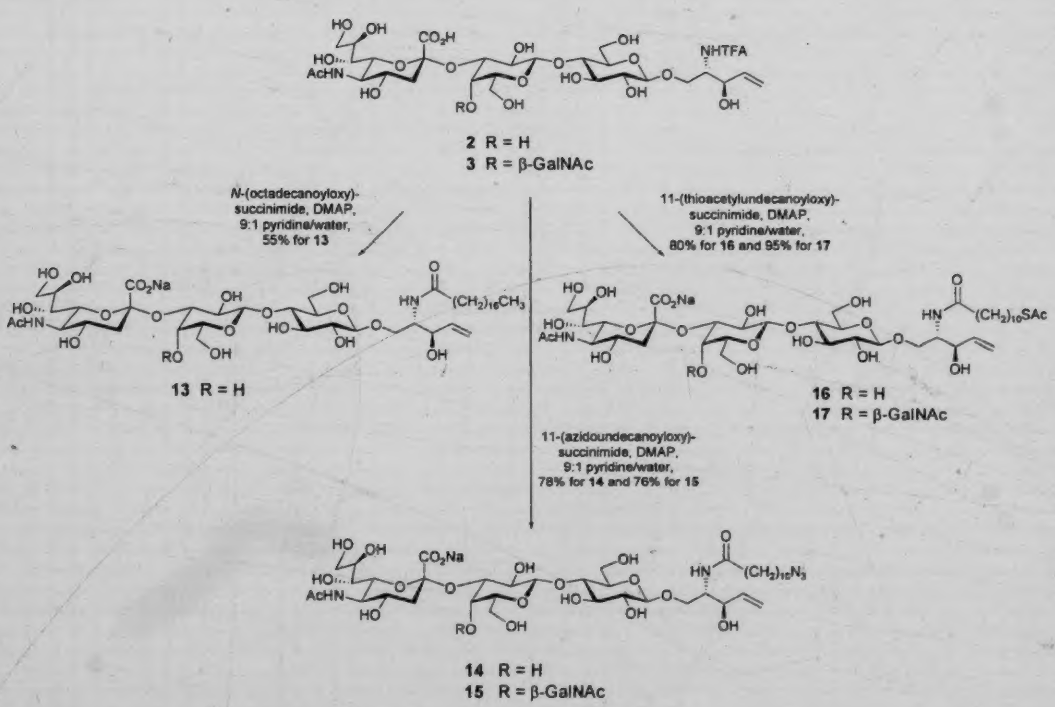
Scheme 2.4 *Chemoenzymatic synthesis of GM_3 and GM_2 analogues* **2** *and* **3**.

---

[2] Current prices of UDP-GalNAc are approximately 20 times that of UDP-GlcNAc.

2.4.3 Derivation of ganglioside analogues with different acyl chains

The immobilization of oligosaccharides on solid surfaces and particles as well as their conjugation to protein are important tools in glycobiology for the study and modulation of carbohydrate functions. Oligosaccharide intermediates bearing a functional group designed for conjugation are valuable compounds for such undertakings. To this end, GM_3 and GM_2 analogues containing a functionalized *N*-acyl chain were synthesized from intermediates **2** and **3** (Scheme 2.5). Deprotection of the sphingosine amine followed by reaction with a succinimide activated octadecanoate yielded compound **13**. It can be envisioned that the long acyl chain could be used to non-covalently coat microtiter plates *via* hydrophobic interactions. Furthermore, following protection, the truncated ceramide could be extended to a natural ceramide *via* olefin metathesis. Alternatively, the alkene portion of the truncated ceramide could serve as a handle for protein conjugation *via* radical addition of cysteamine to the terminal olefin. In an analogous fashion, compounds **11** and **12** were converted to GM_3 and GM_2 mimics **14** and **15**, which contain an azide group at the *N*-acyl chain terminus. These ganglioside analogues could be covalently attached to alkyne-functionalized surfaces *via* a 1,3-dipolar cycloaddition or coupled to proteins or functionalized surfaces upon reduction of the azide to the amine. Similarly, compounds **11** and **12** were converted to **16** and **17**, which bear a terminal thioester. Upon deprotection of the thiol, these molecules would be suitable for attachment to gold surfaces or nanoparticles and for conjugation to proteins.


2 R = H
3 R = β-GalNAc
N-(octadecanoyloxy)-succinimide, DMAP, 9:1 pyridine/water, 55% for 13
11-(thioacetylundecanoyloxy)-succinimide, DMAP, 9:1 pyridine/water, 80% for 16 and 95% for 17
13 R = H
16 R = H
17 R = β-GalNAc
11-(azidoundecanoyloxy)-succinimide, DMAP, 9:1 pyridine/water, 78% for 14 and 76% for 15
14 R = H
15 R = β-GalNAc

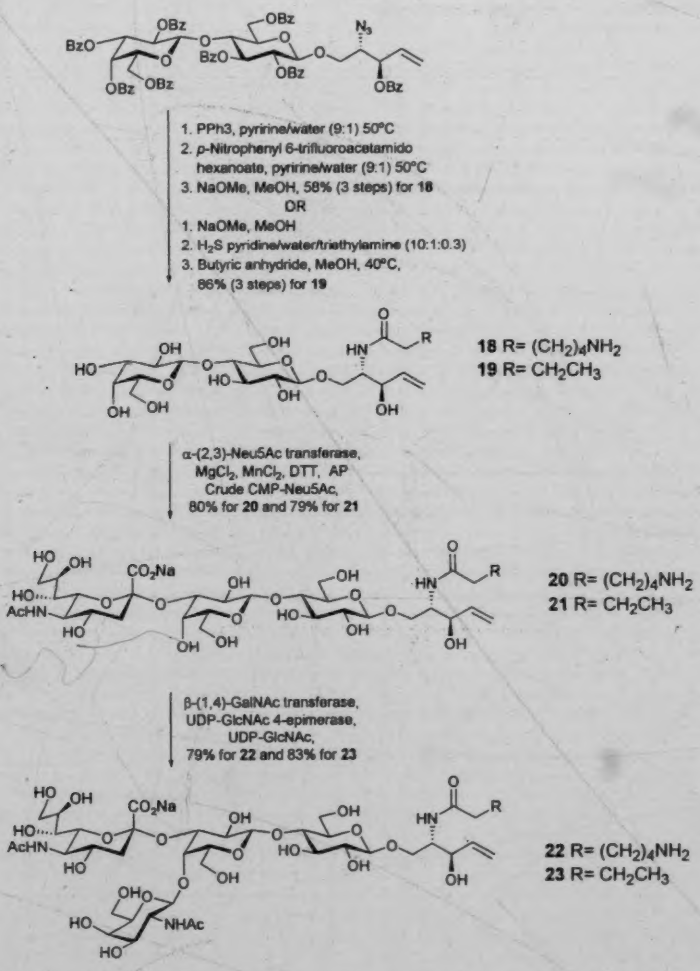
Scheme 2.5 *Derivatization of GM_3 and GM_2 analogues with different acyl chains.*

2.4.4 Alternative Route to Gangliosides[3]

The method described above for accessing GM_3 and GM_2 analogues from a lactose core was particularly useful for the synthesis of compounds incorporating a long hydrophobic chain at the sphingosine nitrogen. However, compounds functionalized with a shorter acyl chain could be synthesized *via* an alternate pathway as shown in Scheme 2.6. As illustrated, the azide group of the benzoylated lactosyl azidosphingosine was first reduced under Staudinger conditions. Acylation of the amine with *p*-nitrophenyl 6-trifluoroacetamido-hexanoate followed by deprotection with sodium methoxide afforded compound **18**. Alternatively, the benzoylated lactosyl azidosphingosine could be deprotected and reduced in the presence of hydrogen sulfide. Reaction of the amine with butyric anhydride afforded **19**. The lactosyl ceramides functionalized with a shorter acyl chain were soluble in aqueous solutions and thus could be used as acceptors in enzymatic glycosylation reactions. Reaction in the presence of α-(2,3)-sialyltransferase then yielded GM_3 analogues **20** and **21**, which could be further elaborated to GM_2 analogues **22** and **23** using β-(1,4)-*N*-acetylgalactosaminyl transferase. While being less versatile than the method described in Section 2.4.2, this stategy allows access to ganglioside analogues incorporating a specific aglycon while minimizing manipulation of the sugar at a late stage of the synthesis.

---

[3] The author would like to thank Jamie Rich for providing some of the compounds described in this section.


1. PPh3, pyririne/water (9:1) 50°C
2. p-Nitrophenyl 6-trifluoroacetamido hexanoate, pyririne/water (9:1) 50°C
3. NaOMe, MeOH, 58% (3 steps) for 18
OR
1. NaOMe, MeOH
2. H2S pyridine/water/triethylamine (10:1:0.3)
3. Butyric anhydride, MeOH, 40°C, 86% (3 steps) for 19
18 R= (CH2)4NH2
19 R= CH2CH3
α-(2,3)-Neu5Ac transferase, MgCl2, MnCl2, DTT, AP Crude CMP-Neu5Ac, 80% for 20 and 79% for 21
20 R= (CH2)4NH2
21 R= CH2CH3
β-(1,4)-GalNAc transferase, UDP-GlcNAc 4-epimerase, UDP-GlcNAc, 79% for 22 and 83% for 23
22 R= (CH2)4NH2
23 R= CH2CH3

Scheme 2.6 *Alternative method for the chemoenzymatic synthesis of GM_3 and GM_2 analogues.*

2.5 Conclusions

A versatile approach to the chemoenzymatic synthesis of GM_2 and GM_3 ganglioside analogues in a form suitable for conjugation has been developed. Our approach was centered on a multifunctional ceramide-type tether designed to facilitate preparation of glycoconjugates *via* a variety of coupling strategies while maintaining the stereochemical and structural features of natural ceramides. This methodology avoids many of the typical problems associated with ganglioside synthesis and provides rapid and efficient access to this important class of molecules. The truncated azidosphingosine moiety confers better reactivity in the glycosylation reaction affording the lactoside product in higher yield and simplifying purification of the intermediates involved. Furthermore, the solubility properties of the truncated lactosyl sphingosine allow for enzymatic elaboration of the oligosaccharide portion of the molecule in a minimal number of steps.

Chapter 3
Preparation of Gangliosides Using the 'Living Factory' Approach

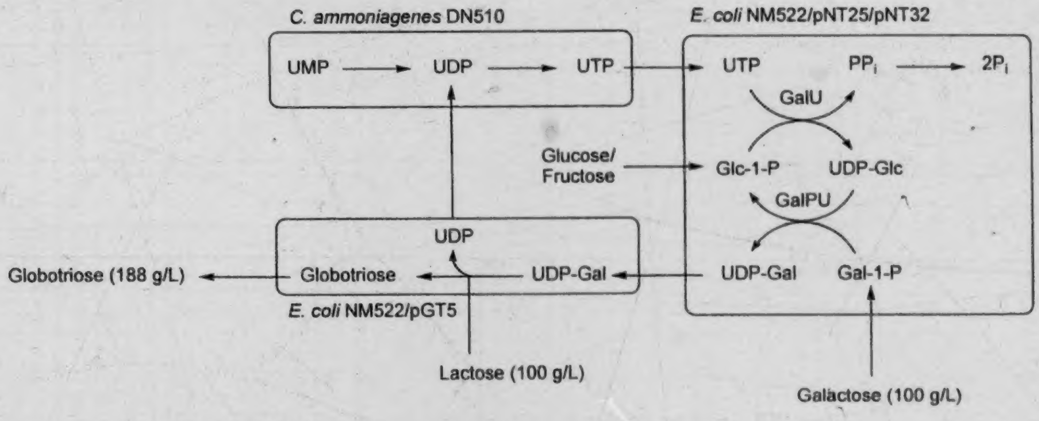
Gangliosides comprise an extended family of glycolipids that appear to play a critical role in virtually every stage of the cell life cycle, including growth, proliferation, differentiation, adhesion, senescence, and apoptosis. As such, gangliosides hold promise for a wide variety of research and therapeutic applications.[190-194] The recent interest in microarray technology,[195-198] heterobifunctional ligands, solid-phase assays, nanoparticles,[199] and conjugate vaccines motivates the preparation of gangliosides in conjugable form. A major limiting factor in the wide application of these glycolipids in disease treatment and research endeavors lies in the great difficulty associated with production of large quantities of pure samples in an efficient and economical fashion. This chapter describes the synthesis and isolation in bacteria living factory of several complex ganglioside analogues including GM_3, GD_3, GM_2, GD_2, GM_1, GD_{1a}, and GT_{1a} from a readily available truncated lactosyl azidosphingosine specially designed for conjugation.

3.1 Oligosaccharide synthesis by metabolically engineered bacteria

Without underestimating the successes obtained from the use of purified glycosyltransferases, the future of oligosaccharide synthesis probably resides in the development of whole-cell biocatalysts. In the past ten years, metabolically engineered bacteria have emerged as a powerful method for the large-scale production of oligosaccharides.[1,62,63,200] This method is based on the production of oligosaccharides in micro-organisms specially engineered to express recombinant glycosyltransferases and the associated biosynthetic enzymes necessary for the recycling of sugar-nucleotide donors. It provides a cost-effective way to produce large quantities of oligosaccharides, avoiding the use of expensive starting materials and the labor intensive isolation and purification of glycosyltransferases. Two different approaches, each with its own benefits and limitations, have been employed to produce oligosaccharides in metabolically engineered bacteria. In the 'bacterial coupling' method, the complication of coupling the central metabolism of the bacteria with oligosaccharide synthesis is avoided by using multiple cells, each with a specialized function (Figure 3.1). This technology takes advantage of a *Corynebacterium ammoniagenes* strain manipulated to efficiently transform a cheap precursor, orotic acid, into UTP. This nucleotide, which accumulates in solution, can be taken up by a second bacterial strain which overexpresses the appropriate biosynthetic enzymes for the production of sugar nucleotide donors. This system can be coupled with a third set of bacteria overexpressing recombinant glycosyltransferases to produce the targeted oligosaccharide in high concentration. While this approach has thus far given the best results in terms of product concentration, the requirements for at least three fermentations for the formation of a single glycosidic bond and the need for permeabilization of the bacteria are less than ideal. On the other hand, in the 'living factory' approach, oligosaccharides are produced in a single growing bacterium that overexpresses all the recombinant glycosyltransferases necessary for construction of the oligosaccharide. The pool of sugar nucleotides is maintained *via* a combination of the bacteria's cellular machinery and engineered biosynthetic pathways (Figure 3.1).

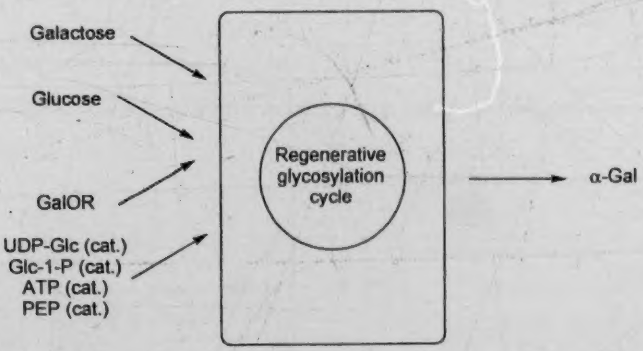
Although yields tend to be lower, the latter strategy is obviously advantageous, requiring the development of a single bacterial strain and producing the target oligosaccharide in a single fermentation step. Several glycosyltransferase genes can be introduced into *E. coli* *via* two different methods. The bacteria can be transformed with several different plasmids, each of which contains a gene for a specific enzyme under the control of compatible promoters.[1, 65-69, 201-205] Alternatively, the microbial strain can be transformed with a single artificial gene cluster incorporating all the biosynthetic genes for the production of the oligosaccharide.[57, 206-209]

A. The 'Bacterial Coupling' Approach



C. ammoniagenes DN510
E. coli NM522/pNT25/pNT32
UMP
UDP
UTP
UTP
PPᵢ
2Pᵢ
GalU
Glucose/
Fructose
Glc-1-P
UDP-Glc
GalPU
UDP
Globotriose (188 g/L)
Globotriose
UDP-Gal
UDP-Gal
Gal-1-P
E. coli NM522/pGT5
Lactose (100 g/L)
Galactose (100 g/L)

B. The 'Living Factory' Approach



Galactose
Glucose
GalOR
UDP-Glc (cat.)
Glc-1-P (cat.)
ATP (cat.)
PEP (cat.)
Regenerative
glycosylation
cycle
α-Gal

Figure 3.1 *(a) Oligosaccharide synthesis by the 'bacterial coupling' approach. (b) Oligosaccharide synthesis by the 'living factory' approach.*

3.2 The 'living factory' approach: Factors to be considered

The microbial synthesis of oligosaccharides is a carbon- and energy-intensive process that poses many challenges in its design. A successful microbial approach is dependent upon a combination of many factors including selection of a host strain in which the biosynthetic enzymes are expressed, generation of a pool of sugar nucleotide donors in the micro-organism, and the intracellular provision of an acceptor.

3.2.1 Host strains

Over the years, diverse oligosaccharide structures have been produced in a variety of host strains including: *Escherichia coli*, *Pichia pastoris*, *Agrobacterium sp*, *Corynebacterium ammoniagenes*, and *Corynebacterium glutamicum*. The choice of the host strain in which the engineered pathway is constructed is crucial to the success of the approach. An efficient system requires the host strain to be capable of simultaneously expressing different glycosyltransferases in active form and at an acceptable level.[62] This can be problematic especially with mammalian glycosyltransferases which are typically difficult to express in bacterial systems.[70] In order to overcome some of the problems associated with the production of oligosaccharides in bacteria, a few avenues using a yeast system (*Pichia pastoris*) have been explored.[210] Yeast systems offer an attractive alternative to the more common bacterial systems as they can produce recombinant oligosaccharides free of any possible endotoxin contamination; an advantage particularly important for the use of these recombinant oligosaccharides in medical applications. However, at present, non-pathogenic strains of *E. coli* are the organisms of choice for the preparation of recombinant oligosaccharides as the availability of well-established genetic tools and commercial vectors greatly simplify the construction of an oligosaccharide-producing microbial factory. Furthermore, under appropriate conditions, *E. coli* can be grown to high or very high cell density (optical density of 100 to 200, in contrast with 2 to 5 under normal conditions) thus maximizing the volume-based productivity.[70]

3.2.2 Sugar nucleotide donors

Sugar-nucleotides are endogenously present in bacteria as a necessary precursor for the synthesis of cell-wall polysaccharides. The sugar nucleotide donors required for oligosaccharide synthesis are the product of many interacting pathways. Their consumption in a foreign biosynthetic pathway applies a tremendous pressure on the central metabolism of the bacteria. Regeneration of sugar nucleotide donors is thus central to the production of oligosaccharides *via* the 'living' factory approach. Different strategies have been devised for the synthesis and recycling of native and unnatural donors in *E. coli*. UDP-glucose and UDP-glucosamine are precursors in the biosynthesis of the bacterial cell wall and all bacteria are capable of their production. While they can be directly used in oligosaccharide synthesis, these cofactors are also important precursors for other sugar nucleotides, such as UDP-galactose and UDP-galactosamine, which can be made *via* a simple epimerization reaction.[206,211,212] More extensive designs are required to produce other sugar nucleotide donors. A system for the production of CMP-Neu5Ac was devised by feeding sialic acid to an *E. coli* strain, in which a CMP-Neu5Ac synthetase gene is overexpressed and the sialic acid aldolase activity abrogated. This system allowed for the synthesis of 17 g/L of CMP-Neu5Ac leading to the high production of sialylactose when coupled to expression of an α-2,3-sialyltransferase.[205] The process can be made even more economical by introducing in the bacteria a pathway allowing for the production of sialic acid from endogenous mannosamine.[213] Another important sugar nucleotide donor in oligosaccharide synthesis is GDP-fucose. The main strategy for its production involves the enhancement of *E. coli* natural production by the overexpression of a positive regulator protein (RcsA) in the colonic acid synthesis pathway in combination with the inactivation of enzymes consuming this donor.[204]

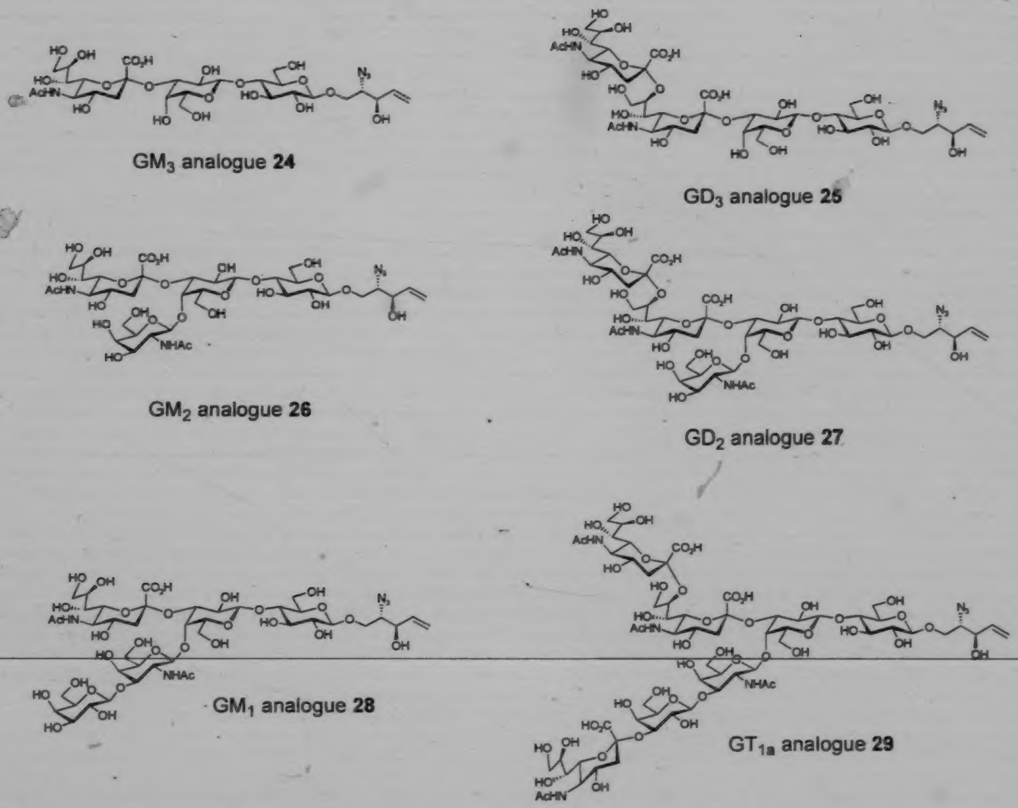
3.2.3 Acceptors

The entry of the acceptor into the cell is a major issue in the design of an efficient method for synthesizing oligosaccharides using metabolically engineered microbes. As the cells selectively uptake an energy source such as glucose, entry of the acceptor into the cells is usually inefficient. Several strategies have been developed to improve uptake of the acceptor while minimizing their degradation. The cell membrane can be permeabilized by freeze-thaw cycles or treatment with various chemicals (detergents, xylene). While these permeabilization techniques are effective, they dissipate the membrane potential necessary for energy metabolism.[62] Alternatively, acceptor sugars can be generated inside the cell by introducing the appropriate enzymes. For example, a system to generate chitin oligosaccharide was developed in which the chitooligosaccharide synthase NodC produced chitotetraose and chitopentaose in *E. coli* cells without any exogenous acceptor. These chitooligosaccharides can in turn serve as an acceptor for glycosyltransferases that recognize a terminal non-reducing *N*-acetylgalactosamine residue.[203] Another very effective method has been devised for introducing the acceptor sugar in cells by manipulating the corresponding catabolite operon. High concentrations of lactose can accumulate intracellularly by deleting the lacZ gene encoding a β-galactosidase responsible for the degradation of lactose while leaving the lactose permease (lacY) gene intact. The synthesis of several valuable lactose-derived oligosaccharides including gangliosides and human milk oligosaccharides was achieved using these methods.[69, 205]

3.3 Results and discussion

3.3.1 Concepts and methodology relating to the production of gangliosides in *E. coli*

Over the past 10 years, the team of Dr. Eric Samain has realized significant advances in the synthesis of the oligosaccharide portion of several gangliosides in living bacteria. They have engineered new biosynthetic pathways in *E. coli*, optimized the conditions for their cultivation at high-cell density and developed a simple isolation and purification procedure for the production of large quantities of gangliosides including GM_3, GD_3, GT_3, GM_2, GD_2, GM_1, GD_{1a}, and GT_{1a}.[68,69,201,205,213] This technology was used to produce several ganglioside analogues incorporating a truncated azido sphingosine aglycon (Figure 3.2).


GM$_3$ analogue **24**

GD$_3$ analogue **25**

GM$_2$ analogue **26**

GD$_2$ analogue **27**

GM$_1$ analogue **28**

GT$_{1a}$ analogue **29**

Figure 3.2 *Targeted ganglioside analogues.*

As previously mentioned in Section 2.1.1, the oligosaccharide portion of gangliosides is centered around a lactose core, which is further elaborated by glycosyltransferases. As the lactose precursor is not endogenous to *E. coli*, a method to introduce the sugar acceptor into the cell was developed. In the absence of glucose, *E. coli* cells can use lactose as a carbon and energy source. The ability of the bacteria to transport and metabolize this sugar relies on the lactose operon, a cluster of tightly regulated genes expressed only in the presence of lactose. The sugar releases a transcriptional repressor allowing for the expression of two genes important in the metabolism of lactose: LacY and LacZ. The LacY gene codes for a β-galactoside permease, a membrane-bound protein which pumps lactose into the cell. This intracellular lactose is then processed by a β-galactosidase, encoded by the LacZ gene, which cleaves the disaccharide into glucose and galactose. Introduction of the sugar acceptor in the engineered cells takes advantage of this catabolic operon.[205] An *E. coli* strain devoid of β-galactosidase activity while still exhibiting full β-galactoside permease activity was developed. Upon induction of the β-galactoside permease gene, lactose is actively internalized by the permease and accumulates intracellularly without degradation to provide a large pool of the sugar acceptor for ganglioside synthesis. We have shown that the selectivity of the β-galactoside permease is flexible enough to accommodate the transport of a lactoside derivative **11** which contains a truncated sphingosine aglycon.

In addition to a lactose acceptor, the biosynthesis of gangliosides in living bacteria requires three different sugar nucleotide donors: UDP-galactose, UDP-*N*-acetylgalactosamine, and CMP-sialic acid. As these sugar nucleotides are not endogenous to *E. coli*, methods for their introduction into the bacteria needed to be developed. All gangliosides are characterized by the presence of one or more sialic acid residues and thus their biosynthesis requires a supply of CMP-sialic acid. Early strategies for the biosynthesis of CMP-Neu5Ac took advantage of the NanT permease.[67,69,205] It is well known that *E. coli* can grow with sialic acid as the sole carbon and energy source due to the presence of a catabolic operon. In the presence of sialic acid, a sialic acid permease (nanT) is expressed permitting the entry of the keto-sugar into the cell. A second gene, nanA is also expressed. This gene encodes an aldolase responsible for the degradation of sialic acid into an energy loaded molecule, pyruvate, and a by-product, *N*-acetylmannosamine. This catabolic pathway was engineered into an anabolic pathway leading to the production of CMP-Neu5Ac. The sialic aldolase activity was inhibited by deleting the nanA gene while the gene for CMP-Neu5Ac synthetase (neuA) was introduced in the cell. This resulted in the generation of an *E. coli* strain capable of producing CMP-Neu5Ac when fed with exogenous sialic acid. As large scale production of gangliosides requires significant quantities of expensive sialic acid, a new bacteria strain, which could produce sialic acid *in situ*, was developed.[213] This microbial system was engineered to produce sialic acid from endogenous UDP-GlcNAc. This strain expresses a GlcNAc-6-phosphate-2-epimerase (neuC) which converts UDP-GlcNAc to ManNAc. Expression of the gene encoding for sialic acid synthase (neuB) in combination with deletion of the genes encoding for ManNAc kinase (nanK) and sialic acid aldolase (nanA) lead to the efficient production of sialic acid. This precursor is in turn processed by CMP-Neu5Ac synthase to generate the required sugar nucleotide donor. All of the genes necessary for the biosynthesis of CMP-sialic acid (neuA, neuB, and neuC) were cloned under the control of the P_{Lac} promoter in a single vector (pBBR1MCS-3)[214] to yield a plasmid coined pBBR3-SS.[213] The synthesis of more complex gangliosides on the other hand, necessitates two additional sugar nucleotide donors: UDP-galactose and UDP-*N*-acetylgalactosamine. These precursors can be obtained by conversion of the endogenous UDP-glucose and UDP-*N*-acetylglucosamine by a bifunctional UDP-GlcNAc/Glc 4-epimerase (gne) isolated from *C. jejuni*.[189] UDP-glucose and UDP-*N*-acetylglucosamine are key metabolites in *E. coli* as precursors for cell wall components necessary for the bacteria viability. UDP-GlcNAc and UDP-Glc are synthesized from fructose-6-phosphate in 4 and 3 steps respectively. Fructose-6-phosphate can be converted to glucosamine-6-phosphate by the enzyme glms. Isomerization by glmM, followed by activation and acetylation by the bifunctional glmU yields the UDP-*N*-acetylglucosamine donor.[215] UDP-glucose is generated in an analogous way. For more complex gangliosides, all of the genes involved in the biosynthesis of the sugar-nucleotide are introduced in the bacteria onto a single plasmid coined pBBR3-SS-gne,[213] a derivative of the pBBR1MCS-3[214] cloning vector encoding the neuA, neuB, neuC, and gne genes. As UDP-Glc and UDP-GlcNAc are substrates for bacterial cell wall biosynthesis, the recombinant epimerase should be expressed at low level so as not to disturb the normal cell processes. This can be achieved by cloning the gene for the epimerase under the regulation of an appropriate promoter such as P_{Lac}. Each strain is then transformed with the appropriate set of glycosyltransferases which allow conversion of lactose to different ganglioside structures under conditions favoring minimal by-product formation. This aspect of the synthesis will be described in detail for each ganglioside derivative in Sections 3.3.2 through 3.3.6.

Having in hand a set of bacteria strains capable of synthesizing different gangliosides, maximum productivity was achieved by growing the engineered bacteria at high-cell density. High-cell density cultures present unique challenges to accommodate the increased nutritional requirements and metabolite production. As most media ingredients become inhibitory at high concentration, simply increasing the concentration of these components is not sufficient to sustain a dense culture.[216] The rapid growth of *E. coli* in the presence of excess glucose can lead to another major problem. As the oxygen delivery capacity of bioreactors is limited, the high density culture of bacteria risks oxygen deprivation, which would oblige the bacteria to shift to a fermentative metabolism, resulting in the production of growth-inhibiting metabolites such as acetic acid.[70, 216] Well-designed media of defined composition coupled with gradual feeding strategies proved to be efficient in tackling these problems. The reduced growth rate induced by supplying the energy source slowly lowers the accumulation of inhibitory metabolites and enables the production of high cell concentration. The most popular feeding strategy for growth of high-cell density cultures is the fed-batch approach. Fed-batch cultures use inocula growing at the maximum specific growth rate that can be sustained using the nutrients initially present in the fermenter. This first growth phase is then followed by various regimes of nutrient feed imposed until fermentation is complete.

The culture of the ganglioside-producing bacteria was carried out under strictly controlled conditions in a bioreactor using a fed-batch approach depicted in Figure 3.3. Culture was initiated in a balanced nutrient medium (HCD-1) that contained all the necessary components for supporting cell growth while avoiding inhibition. In the first phase of the fed batch culture, the bacteria were grown at the maximum rate until complete consumption of the initial supply of glucose. In general this phase lasted for 16 h until optical densities of 25 to 30 were reached. When sufficient bacteria were obtained, the nutrition was switched to an alternative carbon source, glycerol, which was constantly fed to the culture at a rate of 0.15 mL/min. The lower rate of transport of glycerol in the cell leads to a reduction in the flux of carbon into glycolysis, thereby greatly reducing acetic acid formation and favoring entry of the lactose acceptor into the cell. After a short adaptation period, production of ganglioside was initiated. Lactoside **11** and the inducer of the plasmid promoters, isopropyl-β-D-thiogalactopyranoside (IPTG), were added to the culture. The oligosaccharide content of both the extra- and intracellular fractions withdrawn at different cultivation times were analyzed by thin layer chromatography. It was shown that lactoside transiently accumulates into the bacteria before being processed by the appropriate glycosyltransferases.

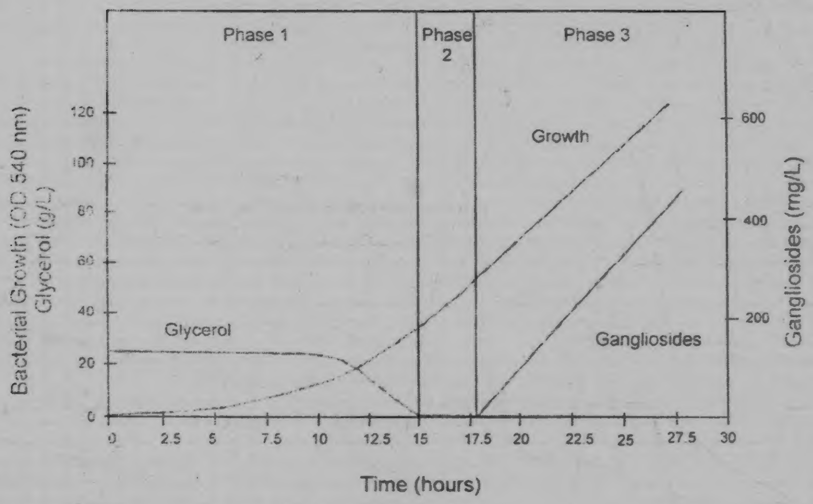


Figure 3.3 *High cell-density culture strategy.*[1]

Upon complete conversion of lactoside **11** into ganglioside, the compounds of interest were isolated and purified. Analysis of both the intracellular and extracellular fractions by thin layer chromatography showed that the target products accumulate intracellularly. To access this fraction, the cells were harvested by centrifugation and permeabilized by heat shock (30 min at 100°C). This treatment enabled the oligosaccharides to diffuse from the cells. After removal of the cellular debris by centrifugation, the proteins were precipitated under acidic conditions to afford an isolate. The purification strategy takes advantage of the Neu5Ac carboxylic acid which confers a negative charge to the molecule. The isolate was further purified by a series of two ion-exchange chromatographies, which separated the negatively charged sugars. Subsequent elution from a size-exclusion column afforded the target compounds in pure form. As a reference, a summary of the gangliosides produced by this method is presented in Table 3.1.

Reaction	Amount of Lactosyl-ceramide Feed to Culture	Amount of Purified Ganglioside Obtained
1	500 mg	GM_3 analogue **24**: 369.9 mg
2	500 mg	GM_3 analogue **24**: 83.3 mg GD_3 analogue **25**: 271.8 mg
3	500 mg	GM_2 analogue **26**: 316.1mg GD_2 analogue **27**: 207.0 mg
4	500 mg	GM_1 analogue **28** : 355.8 mg
5	800 mg	GT1a analogue **29**: 839.9 mg

Table 3.1 *Gangliosides produced using the 'living factory' approach*

3.3.2 Preparation of GM_3 Analogue 24

Ganglioside GM_3 is a ubiquitous metabolite with a variety of cellular activities ranging from control of differentiation, modulation of receptors and tumor association.[84,217-219] As such, GM_3 derivatives amenable for conjugation are important for further biophysical and biological characterization of the processes involving this oligasaccharide. GM_3 analogue **24** was synthesized in an *E. coli* strain (DC7) engineered to express a gene coding for an α-2,3-sialyltransferase. This bacterial strain carries the pBS-nst plasmid that contains an α-2,3-sialyltransferase (nst) from *N. meningitidis* and the pBBR3-SS plasmid that contains the genes coding for a *N*-acetylglucosamine-6-phosphate-epimerase (neuC), sialic acid synthase (neuB), and CMP-Neu5Ac synthase (neuA) allowing for the production of GM_3. The mutant strain that also carried a deletion of both the sialic aldolase (nanA) and *N*-acetylmannosamine kinase (nanK) genes was the most efficient at producing sialylactose derivatives (Figure 3.4).[213]

GM_3 analogue **24** could be synthesized on large scale (500 mg) by cultivating the DC7 strain at high cell density using a fed-batch method described in Section 3.3.1. The cells were grown in a bioreactor under strict regulation of pH and oxygen content in a specially designed high-cell density medium using a pre-determined glucose concentration as an energy and carbon source. Once all the glucose was consumed, the energy source was switch to glycerol to allow optimal entry of the sugar acceptor into the cell. After a short adaptation period, the expression of the β-galactoside permease and the various glycosyltransferase genes was induced by addition of IPTG to the culture media. At this point, the cells were fed lactoside **11** which was rapidly internalized (Figure 3.5, lane 5) and sialylated by the recombinant sialyltransferase using the CMP-Neu5Ac endogenously generated from UDP-GlcNAc (Figure 3.5, lanes 5-7). After four hours the acceptor was completely consumed (Figure 3.5, lane 7).

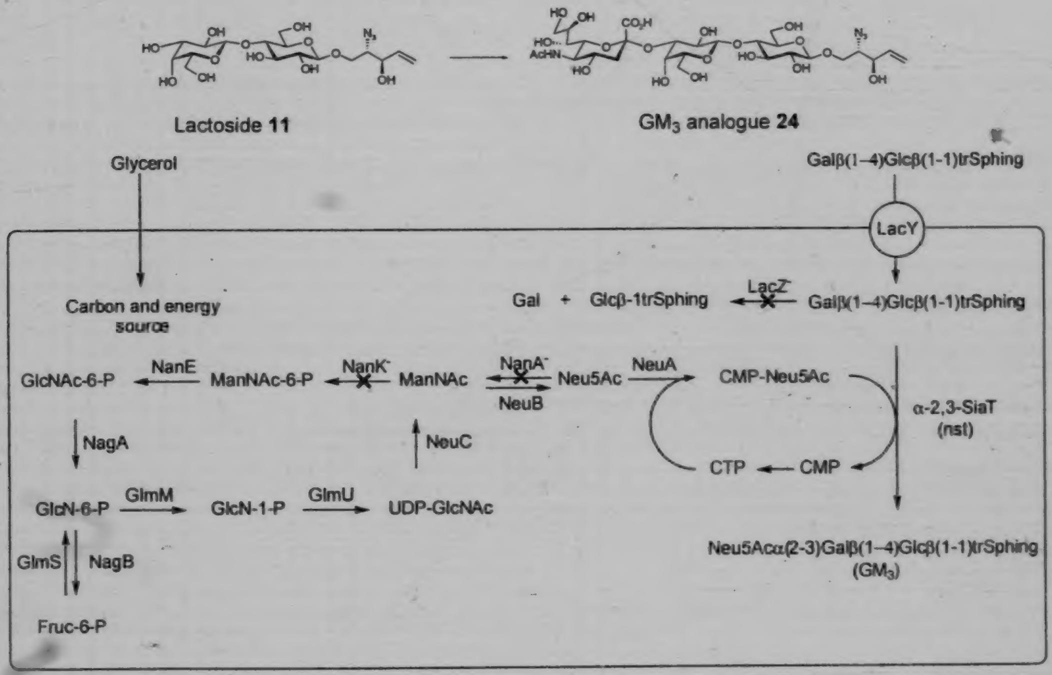

Lactoside **11**
GM₃ analogue **24**
Glycerol
Galβ(1-4)Glcβ(1-1)trSphing
LacY
Carbon and energy source
Gal + Glcβ-1trSphing
LacZ⁻
Galβ(1-4)Glcβ(1-1)trSphing
GlcNAc-6-P
NanE
ManNAc-6-P
NanK⁻
ManNAc
NanA⁻
NeuB
Neu5Ac
NeuA
CMP-Neu5Ac
α-2,3-SiaT (nst)
NagA
NeuC
CTP
CMP
GlcN-6-P
GlmM
GlcN-1-P
GlmU
UDP-GlcNAc
GlmS
NagB
Fruc-6-P
Neu5Acα(2-3)Galβ(1-4)Glcβ(1-1)trSphing (GM₃)

Figure 3.4 *Biosynthesis of* GM_3 *analogue* **24** *by the strain DC7.*

As the oligosaccharides are produced in cell culture, isolation and purification of the product can prove challenging. Analysis of both the intracellular and extracellular fractions by thin layer chromatography showed that the target product accumulates intracellularly. To access this fraction, the cells were harvested by centrifugation and lysed by heat shock. The purification scheme takes advantage of the Neu5Ac carboxylic acid which confers a negative charge to the molecule. Removal of the cellular debris by centrifugation followed by precipitation of the proteins under acidic conditions afforded an isolate which was further purified by a series of two ion-exchange chromatography. Subsequent elution on a size-exclusion chromatography column as described in Section 3.3.1 afforded the target analogue **24** in pure form. From the 500 mg of lactose fed to the culture, 370 mg of GM_3 derivative **24** was obtained after purification and lyophilization. A NMR spectrum of GM_3 derivative **24** is provided in Appendix 1.

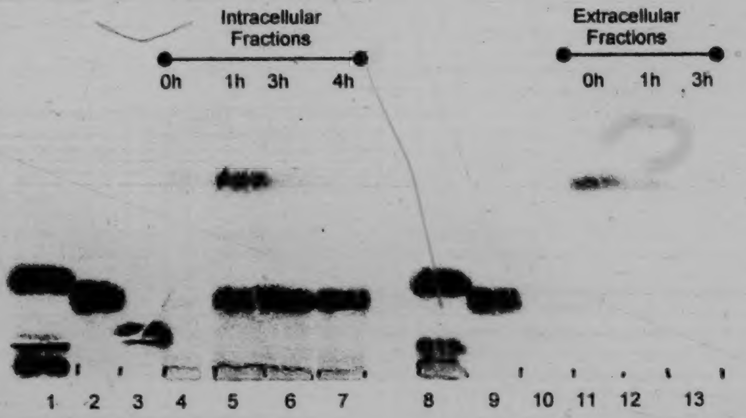


Figure 3.5 *TLC plate analysis of the oligosaccharide content in intracellular (lanes 4-7) and extracellular (lanes 11-14) fractions of samples withdrawn from the culture of GM_3 producing DC7 strain. Standards are in lanes 1 and 8 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 9 (GM_3* **24***) and lanes 3 and 10 (GD_3* **25***). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent.*

3.3.3 Production of GD_3 Analogue 25

GD_3 is a ganglioside found in low concentration in most normal tissues of higher vertebrates. An increase in the level of GD_3 has been correlated with cancer (glioma and melanoma) and has been shown to play an important role in tumor angiogenesis.[220] GD_3 is an attractive target for the treatment of certain cancers as anti-GD_3 monoclonal antibodies have been shown to inhibit the growth of human melanoma cells both *in vitro* and *in vivo*.[221,222] Furthermore, Willison and co-workers have shown that GD_3 immobilized on a solid support depletes anti-ganglioside auto-antibodies from the blood of patients affected by Guillin-Barré Syndrome.[223] Thus, an efficient and economical method to produce this compound is of particular interest for medical applications.

GD_3 analogue **25** was produced from a bacterial strain overexpressing a bifunctional α-2,3/2,8-sialyltransferase (cstII) from *Campylobacter jejuni*[167] as well as the machinery necessary to produce CMP-Neu5Ac[224] *in situ* (Figure 3.6). The bifunctional enzyme catalyzes the transfer of a sialyl moiety from the internally produced CMP-Neu5Ac to the lactoside **11** to form GM_3 analogue **24**. The transfer of a second sialic acid moiety yields the target GD_3 **25**. This reaction can be further extended to produce GT_3, the precursor of all C-series gangliosides.

The bacterial strain was cultured at high cell density as described in Section 3.3.1 in the presence of 667 mg/L of lactoside **11**. Analysis of the extracellular and intracellular fractions by thin layer chromatography revealed that lactoside **11** was rapidly incorporated into the cells (Figure 3.7, lane 4) and almost entirely converted into three products, GM_3 analogue **24**, GD_3 analogue **25**, and trace amounts of a GT_3 analogue (Figure 3.7, lane 5). The ratio of the different oligosaccharides obtained can be controlled by varying the culture time after addition of the substrate and induction of the glycosyltransferases. After collecting the intracellular fraction, the gangliosides were isolated by ion-exchange chromatography as described above, affording separation of the two oligosaccharide fractions, GM_3 and GD_3. The ganglioside analogues were further purified by size-exclusion chromatography to yield 83 mg of GM_3 analogue **24** and 272 mg of GD_3 analogue **25**. NMR spectra for compounds **24** and **25** are provided in Appendix 1.

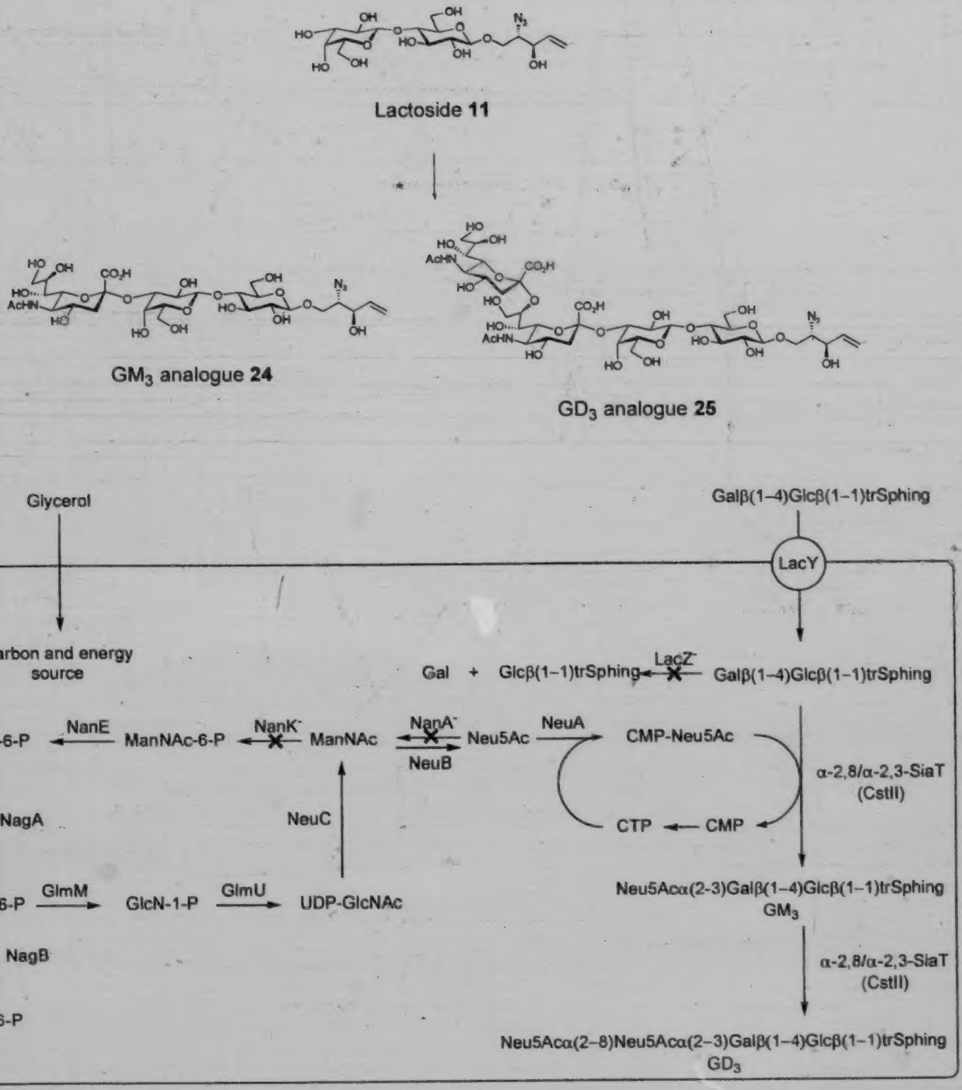

Lactoside 11
GM3 analogue 24
GD3 analogue 25
Glycerol
Galβ(1-4)Glcβ(1-1)trSphing
LacY
Carbon and energy source
Gal + Glcβ(1-1)trSphing
LacZ⁻
Galβ(1-4)Glcβ(1-1)trSphing
GlcNAc-6-P
NanE
ManNAc-6-P
NanK⁻
ManNAc
NanA⁻
NeuB
Neu5Ac
NeuA
CMP-Neu5Ac
CTP
CMP
α-2,8/α-2,3-SiaT (CstII)
NagA
NeuC
GlcN-6-P
GlmM
GlcN-1-P
GlmU
UDP-GlcNAc
GlmS
NagB
Fruc-6-P
Neu5Acα(2-3)Galβ(1-4)Glcβ(1-1)trSphing
GM3
α-2,8/α-2,3-SiaT (CstII)
Neu5Acα(2-8)Neu5Acα(2-3)Galβ(1-4)Glcβ(1-1)trSphing
GD3

Figure 3.6 *Biosynthesis of* GM_3 **24** *and* GD_3 **25** *by strain NF20.*

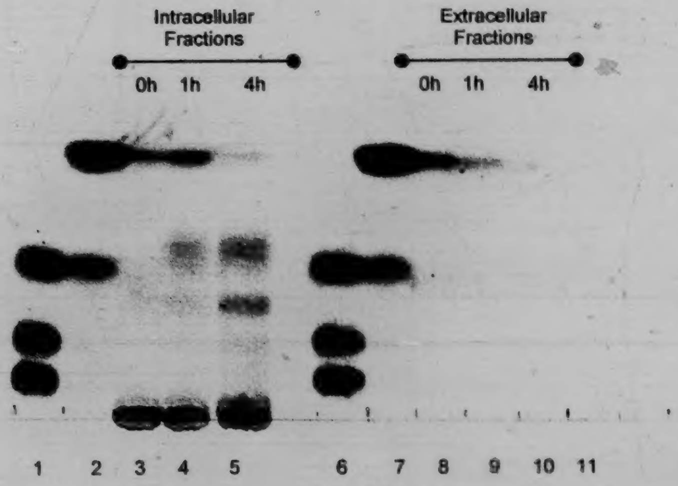


Figure 3.7 *TLC plate analysis of the oligosaccharide content in intracellular (lanes 3-5) and extracellular (lanes 8-10) fractions of samples withdrawn from the culture of* GM_3 *and* GD_3 *producing NF20 strain. Standards are in lanes 1 and 6 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 7 (lactose and lactosyl sphingosine* **11***). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent. A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent.*

3.3.4 Production of GM_2 and GD_2 Derivatives 26 and 27

Oligosaccharides containing the β-D-glycosides of 2-acetamido-2-deoxy hexoses have been widely identified as tumor markers. The system for the production of GD_3 described above can be extended to the production of some members of this important class of tumor-associated antigens.[76] GM_2 analogue **26** and GD_2 analogue **27** can be accessed by a bacterial strain (ZWU12),[213] which expresses, in addition to a bifunctional α-2,3/2,8-sialyltransferase[167] and CMP-Neu5Ac synthase,[224] the genes for a β-1,4-*N*-acetylgalactosaminyl transferase (cgtA)[167] and a UDP-GlcNAc 4 epimerase (gne)[189] from *C. jejuni* (Figure 3.8). The bifunctional α-2,3/2,8-sialyltransferase catalyzes the transfer of a sialic acid residue from the internally generated CMP-Neu5Ac to form GM_3 and GD_3 analogues **24** and **25**. These intermediates are intercepted by the highly active β-1,4-*N*-

acetylgalactosaminyltransferase to form GM_2 **26** and GD_2 **27** derivatives from the UDP-GalNAc produced *in situ*. It has been previously reported that the CgtA β-1,4-*N*-acetylgalactosaminyltransferase also exhibit a β-1,4-galactosyltransferase side activity which results in the formation of a GM_2 analogue designated GA_2 (Figure 3.9).[69] This analogue contains a galactose residue at the non-reducing terminus which can serve as an acceptor for the sialyltransferase to produce a disialylated product, GA_5. This product can in turn be transformed to the pentasaccharides GA_4 and GA_3 by the addition of a galactose or *N*-acetylgalactosamine residue *via* the highly active β-1,4-*N*-acetylgalactosaminyltransferase. The formation of these side products significantly reduces the yield of the target gangliosides by diverting the supply of the sugar nucleotide donors and central intermediates. This undesirable β-1,4-galactosyltransferase activity of the CgtA enzyme can be suppressed by engineering mutants unable to produce UDP-Gal. These mutants can be obtained by disrupting the galU gene which encodes a UDP-glucose pyrophosphorylase involved in the biosynthesis of UDP-Gal.[213] This bacterial strain was used to produce GM_2 and GD_2 derivatives **26** and **27** in a high-density cell culture as described previously (Section 3.3.1).

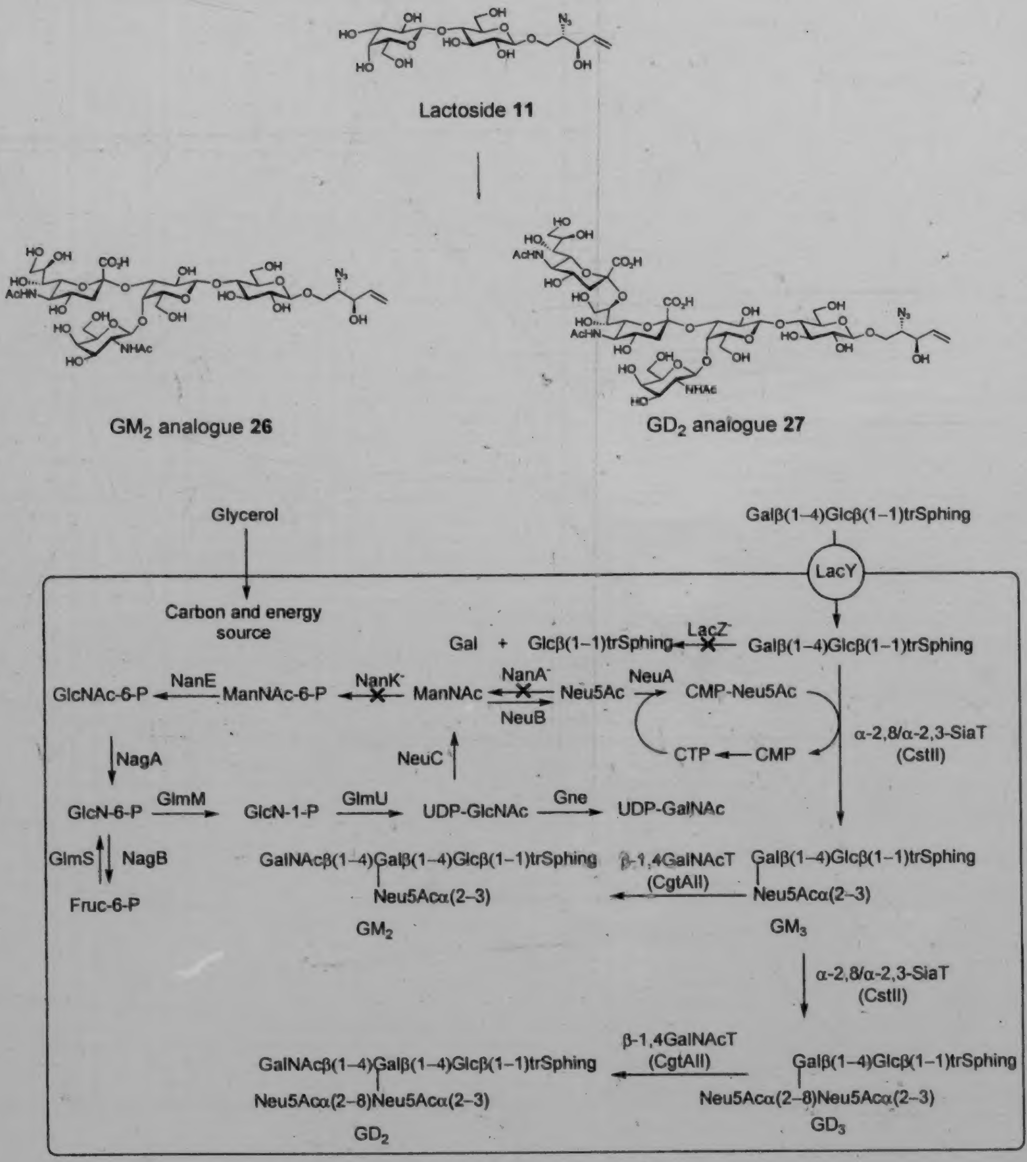

Lactoside 11
GM$_2$ analogue 26
GD$_2$ analogue 27
Glycerol
Carbon and energy source
Galβ(1–4)Glcβ(1–1)trSphing
LacY
Gal + Glcβ(1–1)trSphing
LacZ$^-$
Galβ(1–4)Glcβ(1–1)trSphing
NanE
GlcNAc-6-P
ManNAc-6-P
NanK$^-$
ManNAc
NanA$^-$
NeuB
Neu5Ac
NeuA
CMP-Neu5Ac
CTP
CMP
α-2,8/α-2,3-SiaT (CstII)
NagA
NeuC
GlcN-6-P
GlmM
GlcN-1-P
GlmU
UDP-GlcNAc
Gne
UDP-GalNAc
GlmS
NagB
Fruc-6-P
GalNAcβ(1–4)Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–3)
GM$_2$
β-1,4GalNAcT (CgtAII)
Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–3)
GM$_3$
α-2,8/α-2,3-SiaT (CstII)
β-1,4GalNAcT (CgtAII)
GalNAcβ(1–4)Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–8)Neu5Acα(2–3)
GD$_2$
Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–8)Neu5Acα(2–3)
GD$_3$

Figure 3.8 *Biosynthesis of GM$_2$ and GD$_2$ analogues* **26** *and* **27** *by strain ZWU12.*

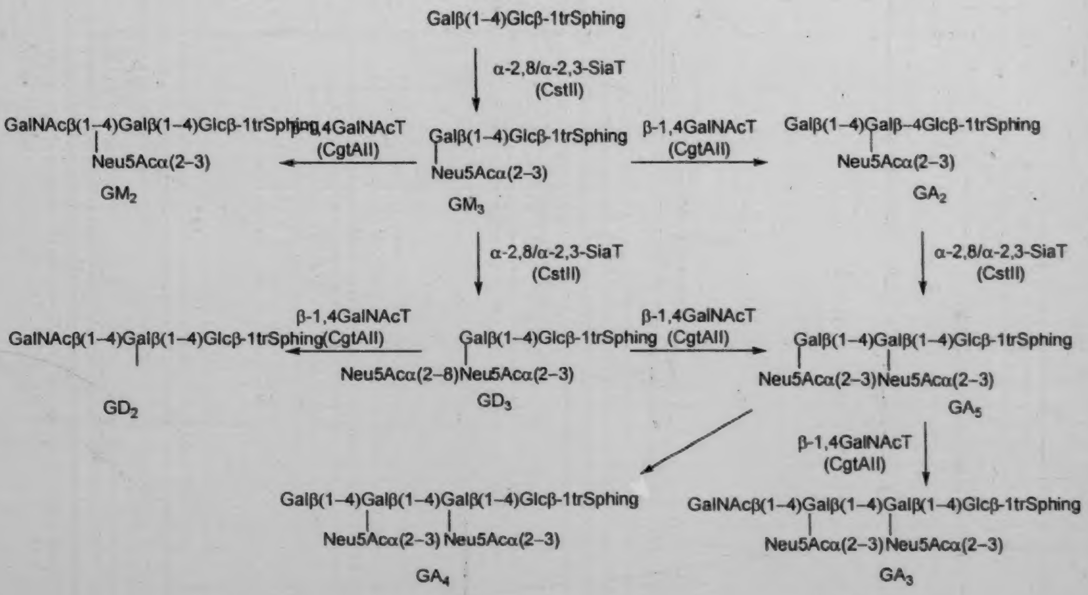

Galβ(1–4)Glcβ-1trSphing
α-2,8/α-2,3-SiaT (CstII)
GalNAcβ(1–4)Galβ(1–4)Glcβ-1trSphing
Neu5Acα(2-3)
GM2
β-1,4GalNAcT (CgtAII)
Galβ(1–4)Glcβ-1trSphing
Neu5Acα(2-3)
GM3
β-1,4GalNAcT (CgtAII)
Galβ(1–4)Galβ-4Glcβ-1trSphing
Neu5Acα(2-3)
GA2
α-2,8/α-2,3-SiaT (CstII)
α-2,8/α-2,3-SiaT (CstII)
β-1,4GalNAcT (CgtAII)
GalNAcβ(1–4)Galβ(1–4)Glcβ-1trSphing
GD2
Galβ(1–4)Glcβ-1trSphing
Neu5Acα(2–8)Neu5Acα(2-3)
GD3
β-1,4GalNAcT (CgtAII)
Galβ(1–4)Galβ(1–4)Glcβ-1trSphing
Neu5Acα(2-3)Neu5Acα(2-3)
GA5
β-1,4GalNAcT (CgtAII)
Galβ(1–4)Galβ(1–4)Galβ(1–4)Glcβ-1trSphing
Neu5Acα(2-3) Neu5Acα(2-3)
GA4
GalNAcβ(1–4)Galβ(1–4)Galβ(1–4)Glcβ-1trSphing
Neu5Acα(2-3) Neu5Acα(2-3)
GA3

Figure 3.9 Possible *by-product formation in the synthesis of GM_2* **26** *and GD_2* **27** *analogues.*

Lactoside **11** (666 mg/L) was added at the beginning of a fed-batch phase along with isopropyl-β-D-thiogalactopyranoside inducing the expression of glycosyltransferases and associated enzymes required for the production of the target oligosaccharides. The truncated lactosyl sphingosine transiently accumulates in the intracellular fraction and is rapidly and completely converted to the targets as shown in Figure 3.10. The desired products are isolated from the intracellular fractions by centrifugation and a series of ion-exchange chromatography steps detailed above. The compounds can then be purified to homogeneity by elution from a size exclusion column. After purification and lyophilization, 316 mg of GM_2 analogue **26** and 207 mg of GD_2 analogue **27** were obtained. Again, the proportion of each product obtained can be adjusted by varying the culture time after induction of the glycosyltransferases. NMR spectra for compounds **26** and **27** are provided in Appendix 1.

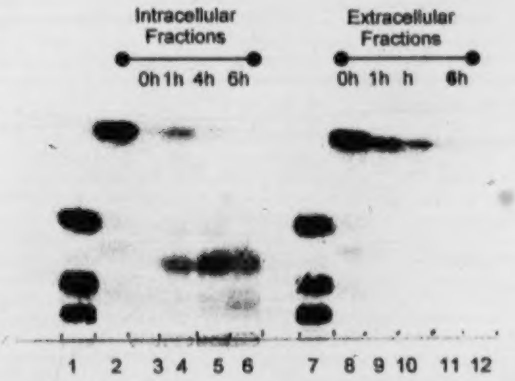


Figure 3.10 *TLC plate analysis of the oligosaccharide content in intracellular (lanes 3-6) and extracellular (lanes 9-12) fractions of samples withdrawn from the culture of GM_2 and GD_2 producing ZWU12 strain. Standards are in lanes 1 and 7 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 8 (lactosyl sphingosine* **11***). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent.*

3.3.5 Production of GM_1 analogue 28

Ganglioside GM_1 is widely expressed throughout the body where it has important physiological functions. In addition to its role in normal physiological processes, GM_1 also play a role in certain disease states. It is a receptor for several bacterial toxins associated with *Escherichia coli* and *Vibrio cholerae* infections.[225,226] Furthermore, GM_1 act as an antigen on the peripheral nervous system for autoantibodies associated with a number of autoimmune neuropathies including Guillain-Barré syndrome.[227] The important physiological roles played by GM_1 motivates the development of tools for its large scale production.

As illustrated in Figure 3.11, a system for the production of GM_1 analogue **28** can be conceived from the system used to produce GM_3. In addition to expressing the α-2,3-sialyltransferase (nst)[228] and all the enzymes necessary for the production of the CMP-Neu5Ac donor *in situ*[224], this bacterial strain expresses the genes encoding a β-1,4-*N*-

acetylgalactosaminyltransferase (CgtAII)[213], β-1,3-galactosyltransferase (CgtB)[167], and a UDP-GlcNAc 4-epimerase (Gne)[189] from *C. jejuni*. In this system, the β-1,4-*N*-acetylgalactosaminyltransferase transfers a *N*-acetylgalactosamine residue from UDP-GalNAc to GM_3 derivative **24** to form GM_2 **26**. Subsequently, the β-1,3-galactosyltransferase transfers a galactosyl moiety to GM_2 affording GM_1 analogue **28**. GM_1 oligosaccharide has a terminal non-reducing galactose residue that can act as an acceptor for α-2,3-sialyltransferase to form GD_{1a} (Figure 3.11). This structure contains the same disaccharide unit as sialylactose and can in turn act as an acceptor for the β-1,4-*N*-acetylgalactosaminyltransferase to form a heptasaccharide intermediate which can be further modified with a galactose residue to form the GA_1 octasaccharide (Figure 3.12). The formation of GD_{1a} and its larger derivatives significantly reduces the yield of GM_1. The formation of these side products can be abolished by introducing in the bacteria a different α-2,3-sialyltransferase (cstIII), which cannot use GM_1 as an acceptor.

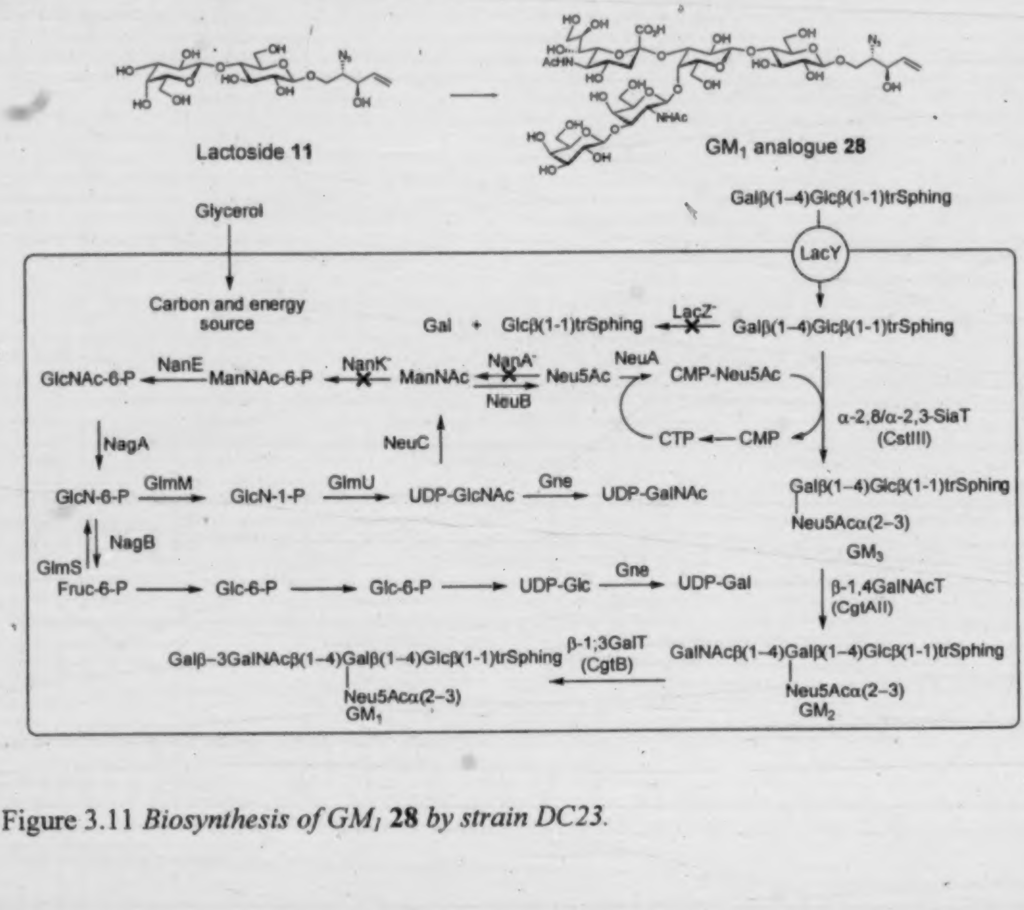

Lactoside 11
GM₁ analogue 28
Glycerol
Carbon and energy source
Galβ(1–4)Glcβ(1-1)trSphing
LacY
Gal + Glcβ(1-1)trSphing
LacZ⁻
Galβ(1–4)Glcβ(1-1)trSphing
GlcNAc-6-P
NanE
ManNAc-6-P
NanK⁻
ManNAc
NanA⁻
NeuB
Neu5Ac
NeuA
CMP-Neu5Ac
CTP
CMP
α-2,8/α-2,3-SiaT (CstIII)
NagA
NeuC
GlcN-6-P
GlmM
GlcN-1-P
GlmU
UDP-GlcNAc
Gne
UDP-GalNAc
Galβ(1–4)Glcβ(1-1)trSphing
Neu5Acα(2–3)
GM₃
NagB
GlmS
Fruc-6-P
Glc-6-P
Glc-6-P
UDP-Glc
Gne
UDP-Gal
β-1,4GalNAcT (CgtAII)
Galβ-3GalNAcβ(1–4)Galβ(1–4)Glcβ(1-1)trSphing
Neu5Acα(2–3)
GM₁
β-1;3GalT (CgtB)
GalNAcβ(1–4)Galβ(1–4)Glcβ(1-1)trSphing
Neu5Acα(2–3)
GM₂

Figure 3.11 *Biosynthesis of* GM_1 **28** *by strain DC23.*

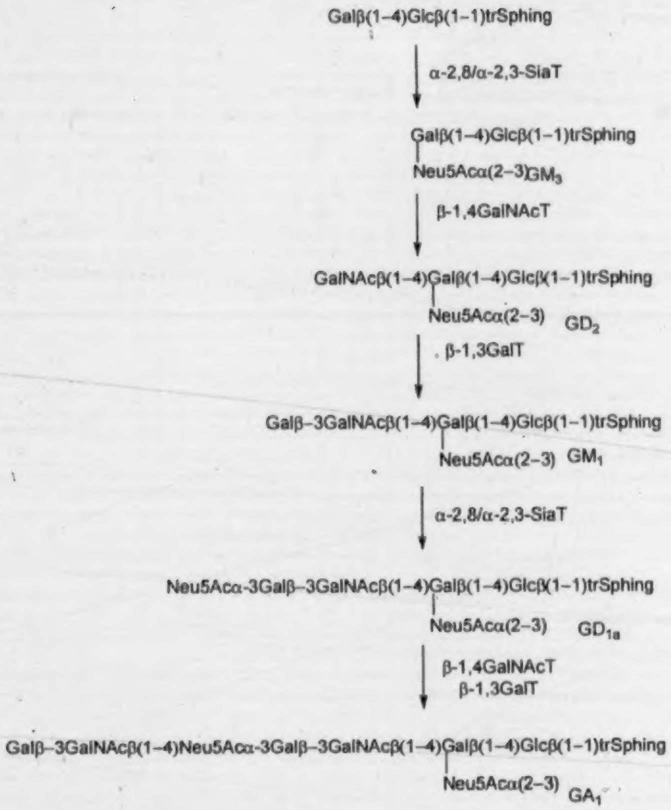

Galβ(1-4)Glcβ(1-1)trSphing
α-2,8/α-2,3-SiaT
Galβ(1-4)Glcβ(1-1)trSphing
Neu5Acα(2-3) GM3
β-1,4GalNAcT
GalNAcβ(1-4)Galβ(1-4)Glcβ(1-1)trSphing
Neu5Acα(2-3) GD2
β-1,3GalT
Galβ-3GalNAcβ(1-4)Galβ(1-4)Glcβ(1-1)trSphing
Neu5Acα(2-3) GM1
α-2,8/α-2,3-SiaT
Neu5Acα-3Galβ-3GalNAcβ(1-4)Galβ(1-4)Glcβ(1-1)trSphing
Neu5Acα(2-3) GD1a
β-1,4GalNAcT
β-1,3GalT
Galβ-3GalNAcβ(1-4)Neu5Acα-3Galβ-3GalNAcβ(1-4)Galβ(1-4)Glcβ(1-1)trSphing
Neu5Acα(2-3) GA1

Figure 3.12 Possible *by-product formation in the synthesis of GM$_1$ analogue* **28**.

This bacterial strain (DC23) was grown at high cell-density in the presence of 500 mg lactoside acceptor **11**. Analysis of the intra- and extracellular fractions by thin layer chromatography (Figure 3.13) revealed that GM$_1$ analogue **28** was produced almost exclusively. After isolation and purification as described in Section 3.3.1, 356 mg of GM$_1$ was obtained. A NMR spectrum for GM$_1$ analogue **28** is provided in Appendix 1.

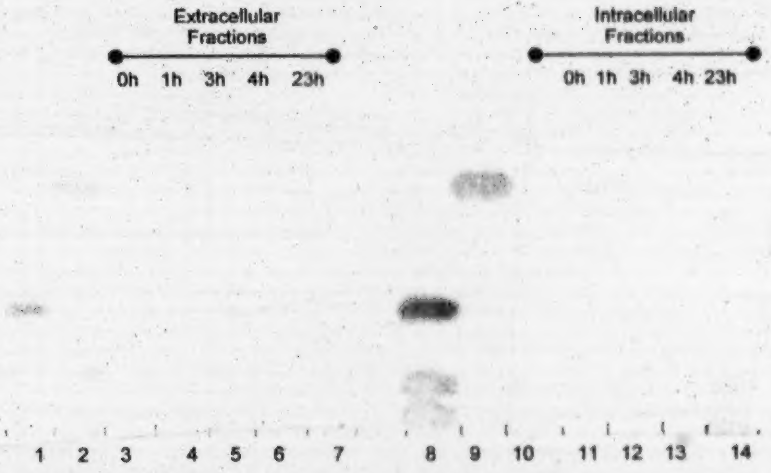


Figure 3.13 *TLC plate analysis of the oligosaccharide content in intracellular (lanes 10-14) and extracellular (lanes 3-7) fractions of samples withdrawn from the culture of GM_1 producing DC23 strain. Standards are in lanes 1 and 8 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose), lanes 2 and 9 (lactosyl sphingosine* **11***). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent.*

3.3.6 Preparation of GT_{1a} Derivative 29

The production GT_{1a} derivative **29** relies on the co-expression of a bifunctional α-2,3/2,8-sialyltransferase (cstII)[167] in combination with a β-1,4-*N*-acetylgalactosaminyltransferase (cgtAII) (
Figure 3.14). The latter enzyme cannot use GD_3 as an acceptor and thus at the end of the culture process only three products are obtained: GD_3, GD_{1a}, and GT_{1a}. The two genes were cloned in the same pBluescript plasmid and were co-expressed with cgtB and the neuABC genes in a nanKEAT mutant. The cstII gene was cloned downstream of cgtA to obtain optimal expression of cgtA with a lower expression of cstII in order to minimize the GD_3 concentration in the final product mixture.[213]

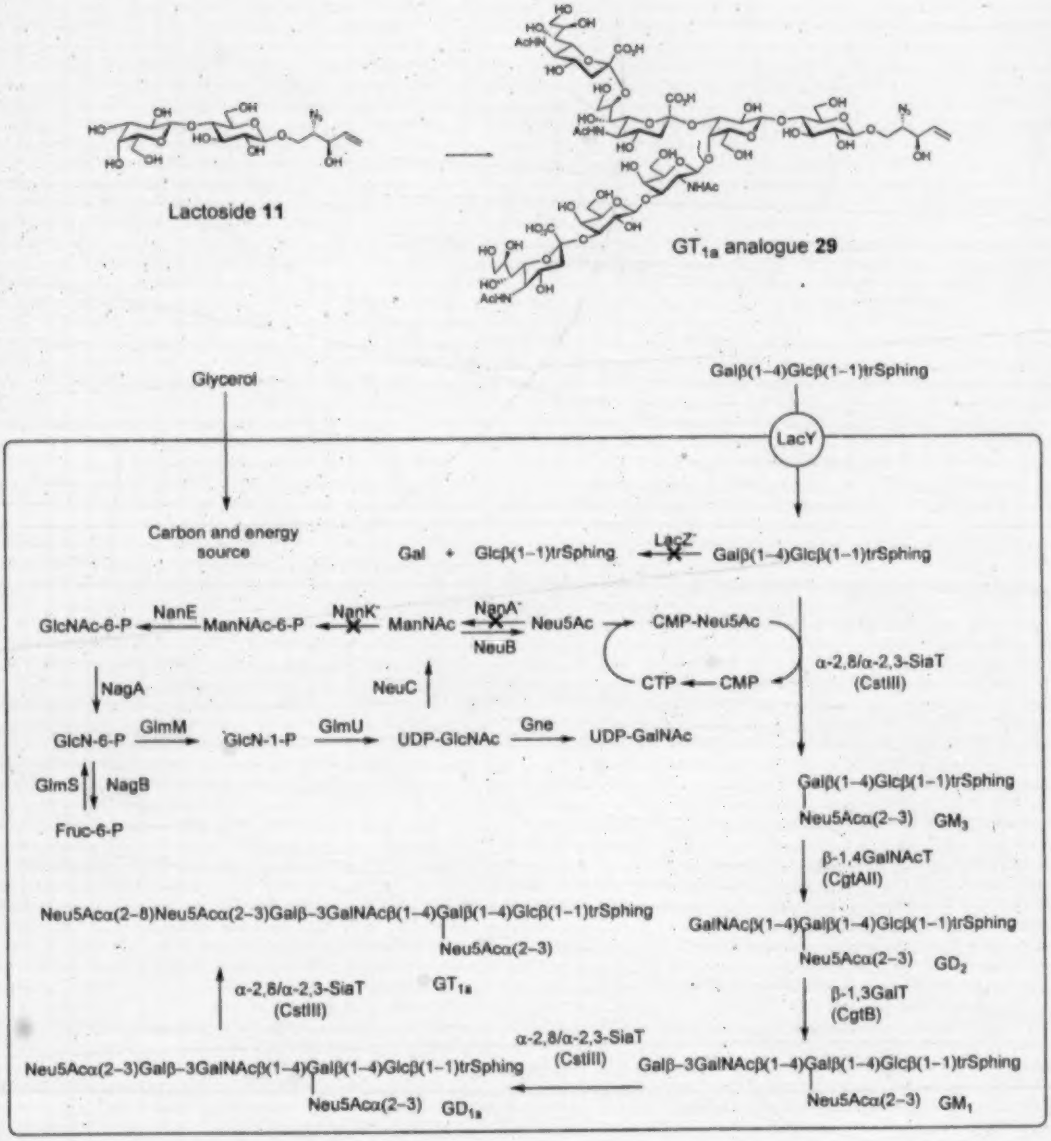

Lactoside 11
GT$_{1a}$ analogue 29
Glycerol
Carbon and energy source
Galβ(1–4)Glcβ(1–1)trSphing
LacY
Gal + Glcβ(1–1)trSphing
LacZ⁻
Galβ(1–4)Glcβ(1–1)trSphing
GlcNAc-6-P
NanE
ManNAc-6-P
NanK⁻
ManNAc
NanA⁻
NeuB
Neu5Ac
CMP-Neu5Ac
CTP
CMP
α-2,8/α-2,3-SiaT (CstIII)
NagA
NeuC
GlcN-6-P
GlmM
GlcN-1-P
GlmU
UDP-GlcNAc
Gne
UDP-GalNAc
GlmS
NagB
Fruc-6-P
Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–3) GM$_3$
β-1,4GalNAcT (CgtAII)
GalNAcβ(1–4)Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–3) GD$_2$
β-1,3GalT (CgtB)
Galβ-3GalNAcβ(1–4)Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–3) GM$_1$
α-2,8/α-2,3-SiaT (CstIII)
Neu5Acα(2–3)Galβ-3GalNAcβ(1–4)Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–3) GD$_{1a}$
α-2,8/α-2,3-SiaT (CstIII)
Neu5Acα(2–8)Neu5Acα(2–3)Galβ-3GalNAcβ(1–4)Galβ(1–4)Glcβ(1–1)trSphing
Neu5Acα(2–3)
GT$_{1a}$

Figure 3.14 *Biosynthesis of GT$_{1a}$ derivative* **29** *by strain NF17.*

This bacterial strain was again cultured at high cell density in a bioreactor as described in Section 3.3.1. At the beginning of the fed-batch phase, the culture was fed with 1.07 g/L of lactoside **11** and expression of the glycosylation system induced by IPTG. After 23 h, the starting material was completely consumed as shown by thin layer chromatography (Figure 3.15), producing a major product which migrated slower that GM$_1$. This oligosaccharide was isolated and purified as described above (Section 3.3.1). Analysis by mass spectrometry demonstrated that the product formed was GT1a. After purification and lyophilization, 840 mg of the GT1a derivative **29** were obtained. A NMR spectrum for compound **29** is provided in Appendix 1.

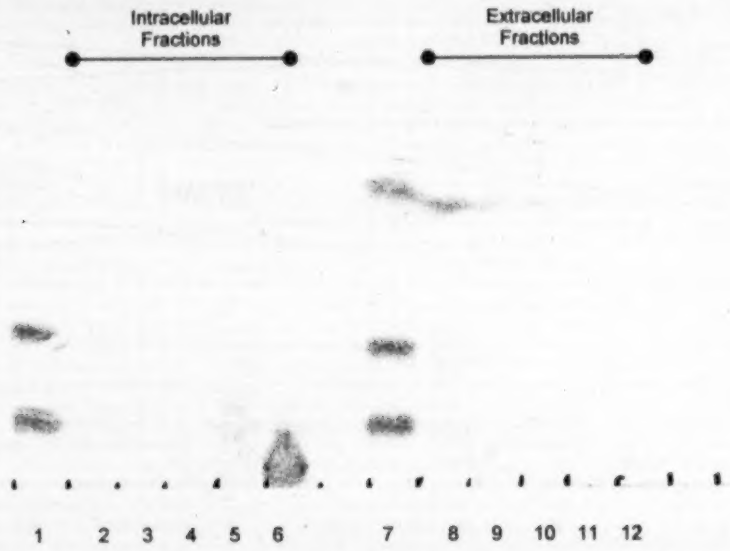


Figure 3.15 *TLC plate analysis of the oligosaccharide content in intracellular (lanes 2-6) and extracellular (lanes 8-12) fractions of samples withdrawn from the culture of GD_{1a} and GT_{1a} producing NF17 strain. Standards are in lanes 1 and 7 (lactose, lacto-N-neotetraose, and lacto-N-neohexanose). A mixture of n-butanol/acetic acid/water (2:1:1) was used as eluent. The sugars were detected using a orcinol/sulphuric acid reagent.*

3.4 Conclusions

Several complex gangliosides were efficiently produced by metabolically engineered *E. coli* strains. These ganglioside structures were prepared from a lactoside acceptor which incorporated a truncated azidosphingosine aglycon. It was demonstrated that this lactoside can be internalized by the engineered bacteria *via* a β-galactoside permease and that the different glycosyltransferases accept this modification to the lipid structure. Gangliosides GM_3, GD_3, GM_2, GD_2, and GT1a were synthesized on a scale of hundreds of milligrams. Following a purification scheme involving ion-exchange and size-exclusion chromatography, these compounds were obtained in homogeneous form, only a few days after initiation of the synthesis from lactoside **11**. These truncated glycolipids incorporated two functionalities which allow for conjugation and immobilization of the gangliosides *via* a wide variety of strategies, rendering these compounds particularly useful for vaccine applications, generation of membrane mimics, and microarrays analyses.

Chapter 4

Evaluation of Ganglioside Conjugates as Cancer Vaccines

4.1 Tumor immunotherapy: significance of gangliosides

Malignant transformation is accompanied by changes in carbohydrate biosynthesis and degradation, resulting in the appearance of altered oligosaccharide motifs at the surface of cancer cells.[74-76] These tumor antigens should, in theory, be recognized as foreign by the immune system and prevent tumor growth.[77] However, suppressive mechanisms permit tumor-presented antigens to be seen as self by the immune system allowing expansion of the tumor.[78,79] Cancer immunotherapy aims to break the immune system's tolerance of tumor antigens as a means to inhibit the growth of cancerous cells. Carbohydrate determinants that are either uniquely expressed or overexpressed on the surface of tumor cells have been the focus of many efforts directed towards the development of cancer immunotherapies.[77] Among many tumor-associated antigens, gangliosides have received considerable attention as targets for therapeutic cancer vaccines.[86,88] This class of glycolipids is highly overexpressed in melanoma and other tumors of neuroectodermal origin, making these structures interesting targets for active cancer immunotherapy.[77,184,229]

Evidence in cancer patients has shown that natural or passively administered anti-ganglioside antibodies are associated with a more favorable prognosis and a prolonged disease-free period.[90,230] These observations have motivated the development of ganglioside-based vaccines as a viable approach in the treatment of cancer. Gangliosides, like the majority of carbohydrate antigens, induce an immune response primarily restricted to the production of antibodies. While the induction of a cell mediated response which can trigger apoptosis of transformed cells is particularly desirable to abolish tumors, antibody production also has its place in the context of cancer immunotherapy.[231] A humoral immune response is particularly well-suited for the elimination of circulating tumor cells and micrometastases *via* antibody-dependent cell-mediated cytotoxicity.[88] If, upon immunization, antibodies of sufficient titer can be induced against cell surface tumor antigens, cancerous cells could be eliminated from the vascular and lymphatic circulation and metastases could be inhibited.[88] This strategy would profoundly alter cancer treatment and permit the use of less invasive approaches to prevent metastasis in patients.

Gangliosides, in particular GM_3, GM_2, GD_3 and GD_2, have generated significant interest for use as immunogens in the framework of active cancer immunotherapy.[77] Unfortunately, these tumor-associated antigens are poorly immunogenic, making their use as immunogens impractical and the preparation of anti-ganglioside antibodies for passive immunization difficult. Gangliosides are poor immunogens because they are self-antigens normally present in the body.[77] Thus, strategies for increasing the immunogenicity of these tumor-associated glycolipids involve their inclusion in the context of foreign antigens for presentation to the immune system. Conjugation of an oligosaccharide epitope to an immunogenic carrier protein has been widely used as a strategy to enhance the immunogenicity of otherwise T-cell independent carbohydrate antigens[232] and has been applied to augment the immunogenicity of gangliosides.[233] Indeed, immunization with a GM_2-KLH conjugate in the presence of adjuvant induced IgM antibodies of moderate titer and a partial class switch to IgG antibodies was observed. Thus far, vaccines based on naturally occurring gangliosides or semi-synthetic gangliosides conjugated to keyhole limpet hemocyanin (KLH) have displayed only limited success in clinical trials.[86, 184-186] These results have motivated new strategies for the design of ganglioside-based vaccines in order to improve their immunogenicity.

4.2 Humoral immune response: Factors to consider

Evaluation of an antibody response encompasses different variables including the magnitude, specificity, affinity and isotype of the immunoglobulin response. The initial recognition event in the humoral immune response involves binding of the antigen to the immunoglobulin receptor at the surface of naïve B-cells of appropriate specificity. This recognition event stimulates the proliferation and differentiation of the selected group of B-cells, and leads ultimately to the generation of an antigen-specific antibody response.

A given antigen can interact with different B-cells of the repertoire, resulting in the generation of a multitude of distinct antibodies against that particular antigen. This phenomenon is of particular importance in the design of glycoconjugate vaccines as the glycoconjugate is likely to stimulate secretion of antibodies not only against the desired carbohydrate epitope, but also against the surface of the carrier protein and potentially any linker. As a result, the immune sera consists of a heterogenous mixture of antibody paratopes, each having a different specificity for a distinct epitope of the immunogen. The complexity of the humoral immune response is further enhanced by the presence of different antibody isotypes. The isotypic variation of the antibody response stems from the nature of the antigen and its route of administration and is an important determinant of the physiological effects of the antibody. In the context of glycoconjugate cancer vaccines, two isotypes are of particular interest: IgM and IgG. The IgM isotype is the first type of antibody secreted in response to an immunogen. It is characterized by a inherently low affinity for the antigen which is somewhat compensated for by the high avidity of its pentameric nature.[73] The persistence of the antigen in the animal or subsequent re-exposure of the animal to the same antigen results in two interlinked processes, class-switch recombination and affinity maturation, ultimately leading to the generation of IgG antibodies of higher affinity.[73] The higher affinity of IgG antibodies leads to a more effective elimination of the target cells compared to IgM antibodies. This former class of antibodies is thus desired in most therapeutic vaccination strategies.

4.3 Immunization: Experimental considerations

The immunogenicity of a glycoconjugate vaccine in a given animal model is dependent on numerous variables including the nature of the glycoconjugate itself and its mode of delivery which may dramatically impact the magnitude of the response, the isotype of antibodies produced and their ability to bind and eliminate the intended target. It is thus of considerable importance to take into account many factors in the design of a glycoconjugate construct for vaccination purposes. Notably, the nature of both the oligosaccharide and carrier protein, the conjugation strategy, the number of haptens incorporated into the construct and its availability determine the extent of the immune response to a particular construct.

4.3.1 Carrier protein

Tumor immunotherapy typically targets oligosaccharide antigens exclusively expressed or highly overexpressed at the surface of cancer cells. On their own, most of these antigens generate thymus-independent responses resulting in the production of low affinity IgM antibodies and the absence of immunological memory as discussed in Sections 1.4 and 4.2. The low immunogenicity of oligosaccharides can be augmented by coupling the carbohydrate antigen to an immunogenic carrier protein providing peptide epitopes for T_H-lymphocyte activation. The choice of a carrier protein is determined by its immunogenic properties, toxicity, stability, and the presence of functional groups for conjugation.[234] The primary role of the carrier protein is to confer immunogenicity to the hapten of interest. The carrier protein should therefore be foreign to the immunized animal and should not be glycosylated. As the result of any immunization strategy is difficult to predict, the carrier protein is often chosen from a group of well-studied models and the ultimate selection is often based on the results of related studies. Common carrier proteins include: keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), tetanus toxoid (TT) and diphtheria toxoid (DT).[234] Good immunological results for tumor-antigens conjugated to KLH have popularized the use of this carrier protein in tumor immunotherapy studies.[77,88]

Despite its ability to confer high immunogenicity to oligosaccharide haptens, KLH is not the ideal carrier protein. KLH is a very large multi-subunit protein that exists in many multimeric forms and is affected by solubility and stability problems.[234] As a result, these glycoconjugate preparations are often heterogeneous and determination of the number of hapten incorporated is complicated by the large size of the protein. It is thus reasonable to infer that approval of such constructs for clinical applications by regulatory agencies would be difficult. Tetanus toxoid has proved to be a good carrier protein in many conjugate vaccines aimed at preventing bacterial and fungal infections.[235-239]

Furthermore, the administration of tetanus toxoid is already approved for use in humans as part of a vaccine against tetanus. Although this protein can also form multimeric aggregates, the different forms can be readily separated by size-exclusion chromatography to provide the monomeric subunit, which may be stored in solution with minimal aggregation. TT monomer is more easily handled than KLH as it is more soluble in buffers and its size is amendable to analysis by mass spectrometry. These properties of tetanus toxoid motivated its selection as a carrier protein for the study presented herein.

4.3.2 Conjugation strategy

The mode of coupling the oligosaccharide hapten to the carrier protein has an important impact on the nature of the final conjugate and its immunogenicity. It impacts the hapten-to-carrier ratio which in turn may affect the immune response to the conjugate. It is generally accepted that higher hapten incorporation levels confers a greater hapten-specific antibody response provided that peptide motifs on the carrier protein essential for T_H cell recruitment are not masked.[240,241] Typical strategies for the generation of a conjugate vaccine involve the coupling of a carbohydrate antigen *via* a linker to reactive groups at the surface of a carrier protein. A wide range of homo- and heterobifunctional linkers have been used to effect this process including succinimide ester derivatives, maleimide derivatives, diethyl squarate, azides and alkynes.[234] Although couplings *via* these methodologies are often highly efficient, they introduce immunogenic structures into the glycoconjugate that can divert the immune response from the oligosaccharide hapten.[242-244] Homobifunctional linkers derived from adipic acid have found multiple applications in the preparation of dendrimers and conjugates. The *p*-nitrophenyl adipate diester derivative has found most use in bioconjugate applications as this derivative has demonstrated a good balance of stability and reactivity.[245] This allows for activation of an amine-functionalized oligosaccharide in reasonable yield, purification of the half-ester intermediate, and efficient coupling to a carrier protein. This linker introduces flexibility in the conjugate in the form of a 6-carbon spacing between the oligosaccharide hapten and the carrier protein, allowing for a better presentation of the hapten at the surface of the carrier. Furthermore, the aliphatic nature of the linker minimize the formation of antibodies against its structure.[242-244] These properties make an adipate-based linker particularly well-suited for use in cancer vaccines.

4.3.3 Immunization protocol

The properties of the immunogen alone are not sufficient to determine the success of an immunization. The success of an immunization also depends on properties of the biological system that the antigen encounters including the dose and route of antigen administration, and adjuvants.[73] The dose of antigen administered has an important influence on immunization as an insufficient or an excessively high dose is likely to induce immunological tolerance. Maximum responses are usually obtained by repeated administration of the antigen over a certain period. Such booster immunizations increase the clonal proliferation of antigen-specific B- or T-lymphocytes and thus increase the lymphocyte populations specific for the immunogen. Immunogens are typically administered *via* the following routes: intravenous, intradermal, subcutaneous, intramuscular and intraperitoneal. The route of administration influences the cell populations involved in the response as the antigen reaches different organs at different rate depending on the site of injection. Typical immunization protocols make use of adjuvant to enhance the immunogenicity of antigens. It is believed that adjuvants augment the immune response by prolonging the persistence of the antigen, enhancing co-stimulatory signals and inflammation, and stimulating the nonspecific proliferation of lymphocytes. Several adjuvant have been developed for use in immunization including the potent Freund's adjuvants which are commonly used in animal studies.

4.4 Results and discussion

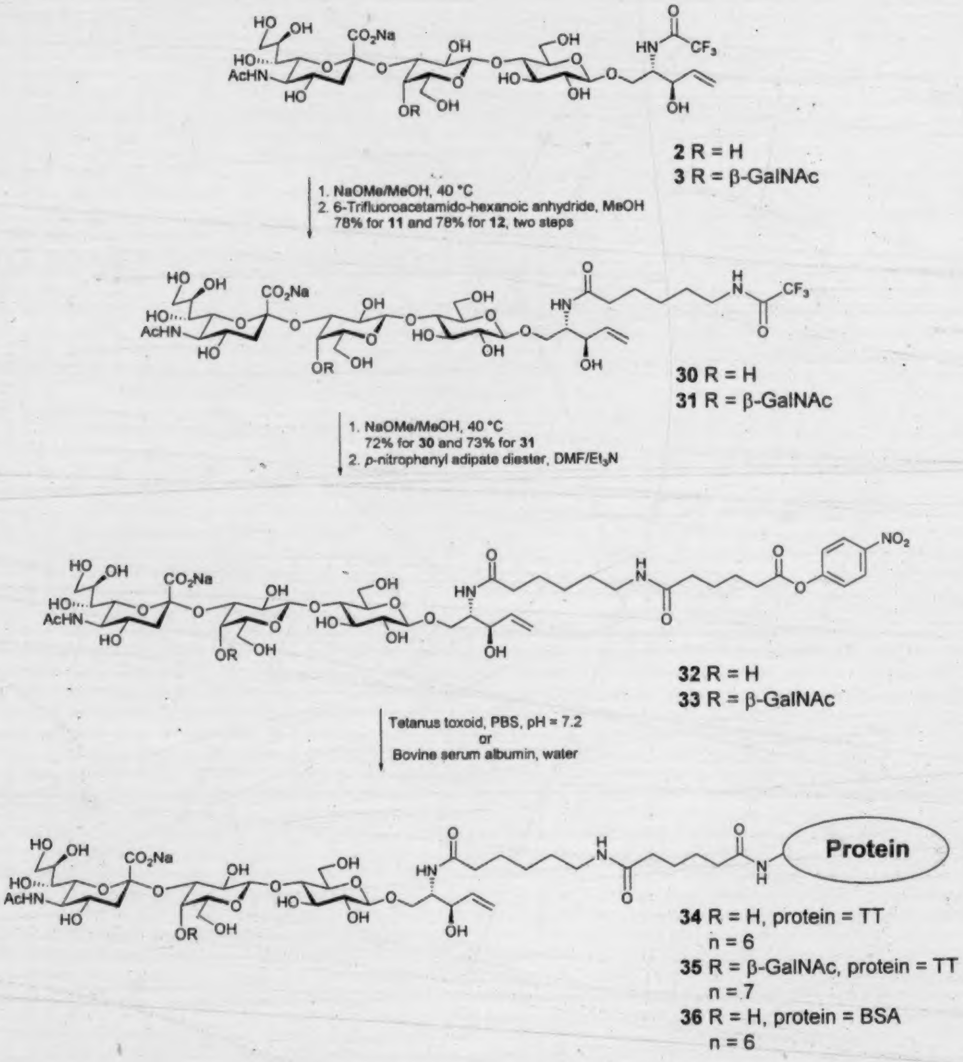
4.4.1 Synthesis of ganglioside conjugates and inhibitors for immunization study

4.4.1.1 Preparation of ganglioside conjugates

The use of tetanus toxoid in commercial carbohydrate vaccines and recent success in the Bundle group in inducing high antibody titers against tetanus toxoid glycoconjugates motivated the use of this carrier protein in the study presented herein.[235-239] As mentioned earlier, the generation of a conjugate vaccine involves the coupling of an oligosaccharide hapten to a reactive group at the surface of the carrier protein. Ideally, the coupling chemistry should allow for appropriate levels of substitution while using a minimal amount and maintaining the structural integrity of precious oligosaccharide haptens. To this end, an adipate-based linker was chosen to effect conjugation of the ganglioside haptens without introducing a potentially immunogenic tether.[245]

The preparation of GM_3 and GM_2 conjugates first involved the introduction of an amine terminated spacer unit. Treatment of general intermediates **2** and **3** presented in Chapter 2 with sodium methoxide in methanol yielded the corresponding amines. *N*-Acylation with 6-trifluoroacetamido-hexanoic anhydride afforded intermediates **30** and **31**. Following deprotection of the primary amine, the gangliosides were activated for conjugation to proteins. Reaction of amino terminated gangliosides with *p*-nitrophenyl adipate diester in a mixture of *N*,*N*-dimethylformamide and triethylamine followed by reverse phase HPLC purification in the presence of 1% acetic acid afforded activated ganglioside analogues **32** and **33** for protein coupling. Incubation of tetanus toxoid with approximately 30 equivalents of the activated sugars yielded glycoconjugates **34** and **35** with average incorporation levels of 6 and 7 respectively as determined by MALDI-TOF mass spectrometry (Scheme 4.1).

The tetanus toxoid glycoconjugates **34** and **35** were used to immunize mice and their ability to raise an immune response was evaluated by ELISA. Synthetic glycoconjugates possessing a heterologous protein were required to detect carbohydrate specific antibodies by solid phase assays (ELISA). For this purpose, ganglioside analogue **32** was coupled to bovine serum albumin using a similar approach to the one used to generate tetanus toxoid conjugates (Scheme 4.1). A hapten incorporation level of 6 was obtained for the GM_3-bovine serum albumin conjugate. In order to perform a more stringent assay for carbohydrate specific antibodies and to exclude the possibility of tether specific antibodies, we sought a glycoconjugate consisting of the same carbohydrate hapten conjugated to a heterologous protein *via* a tether distinct from the one used in the immunizing agent. Bovine serum albumin conjugates containing a squarate linker were prepared from intermediates **30**, **31**, **37**, **38**, and **39** to address this requirement.



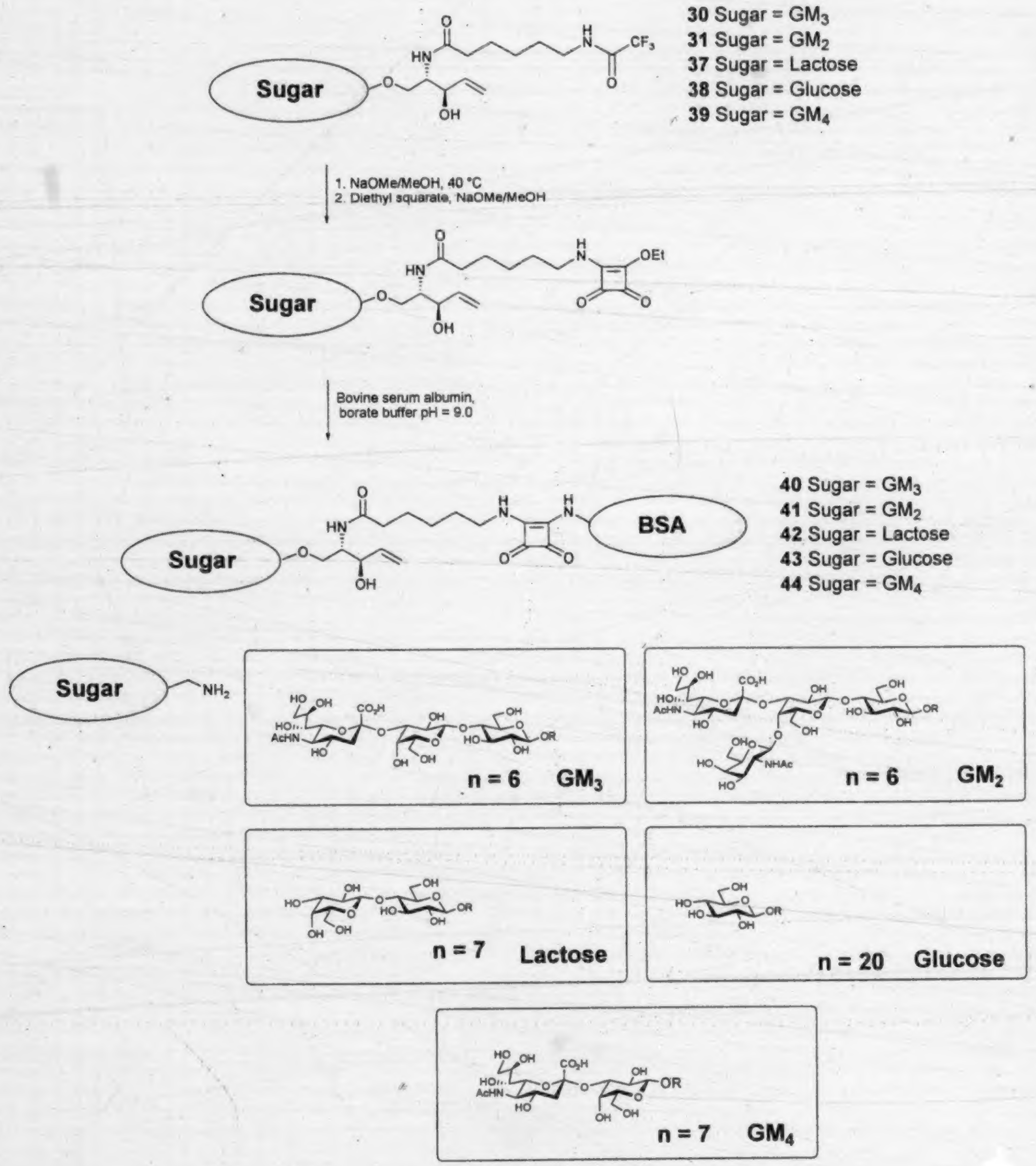
2 R = H
3 R = β-GalNAc
1. NaOMe/MeOH, 40 °C
2. 6-Trifluoroacetamido-hexanoic anhydride, MeOH
78% for **11** and 78% for **12**, two steps
30 R = H
31 R = β-GalNAc
1. NaOMe/MeOH, 40 °C
72% for **30** and 73% for **31**
2. *p*-nitrophenyl adipate diester, DMF/Et_3N
32 R = H
33 R = β-GalNAc
Tetanus toxoid, PBS, pH = 7.2
or
Bovine serum albumin, water
Protein
34 R = H, protein = TT
n = 6
35 R = β-GalNAc, protein = TT
n = 7
36 R = H, protein = BSA
n = 6

Scheme 4.1 *Preparation of tetanus toxoid and bovine serum albumin conjugates using an adipate linker.*

In order to screen immune sera reactivity, different fragments of the oligosaccharide portion of the gangliosides were conjugated to bovine serum albumin *via* a squarate linker (Scheme 4.2).[4] Oligosaccharide fragments spanning different portions of the glycolipid (**30**, **31**, **37**, **38**, and **39**) were first activated by reaction with diethyl squarate in methanol at pH 9.0. After evaporation of the solvent and resuspension of the residue in borate buffer, pH = 9.0, bovine serum albumin was added to the solution to effect conjugation. Extensive dialysis against milliQ filtered water followed by lyophilization afforded BSA conjugates **40**, **41**, **42**, **43**, and **44** with incorporation levels of 6, 6, 7, 20, and 7 respectively.

---

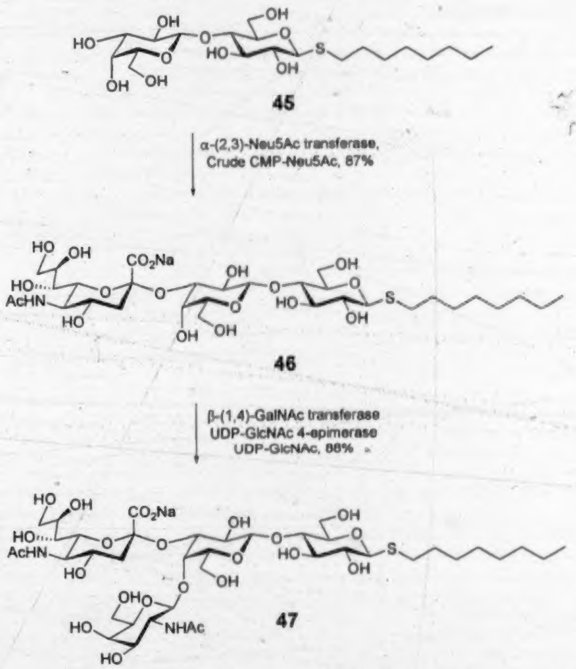
[4] The author would like to thank Mr. Jamie Rich for the preparation of some of these conjugates.


30 Sugar = GM3
31 Sugar = GM2
37 Sugar = Lactose
38 Sugar = Glucose
39 Sugar = GM4
Sugar
HN
O
OH
CF3
1. NaOMe/MeOH, 40 °C
2. Diethyl squarate, NaOMe/MeOH
OEt
Bovine serum albumin,
borate buffer pH = 9.0
BSA
40 Sugar = GM3
41 Sugar = GM2
42 Sugar = Lactose
43 Sugar = Glucose
44 Sugar = GM4
NH2
n = 6 GM3
n = 6 GM2
n = 7 Lactose
n = 20 Glucose
n = 7 GM4
CO2H
AcHN
NHAc
OR

Scheme 4.2 *Preparation of bovine serum albumin glycoconjugates with squarate.*

4.4.1.2 Synthesis of soluble ganglioside inhibitors

The relative binding affinities of different ganglioside fragments can be obtained in a direct ELISA assay in which the sera reactivity is screened against a panel of different immobilized antigens. Results of this assay can be further confirmed by a competitive ELISA assay which will be described in detail in Section 4.4.2.3. Competitive ELISA assays require a panel of soluble oligosaccharides incorporating different elements of the immunogen. In order to test the importance of the truncated ceramide moiety on antibody binding, a set of oligosaccharides bearing an aliphatic aglycon was required. GM_3 and GM_2 oligosaccharides bearing a *S*-octyl chain as a aglycon were thus synthesized using the chemoenzymatic approach we developed (Scheme 4.3). The acceptor, *S*-octyl lactose was first treated with an α-(2,3)-sialyltransferase in the presence of CMP-sialic acid to afford GM_3 derivative **46** in 87% yield. This derivative was further transformed to GM_2 *via* incubation of the oligosaccharide with β-(1,4)-*N*-acetylgalactosaminyl transferase, UDP-*N*-acetylglucosamine-4-epimerase and UDP-*N*-acetylglucosamine to give the target compound **47** in 88% yield.



Scheme 4.3 *Synthesis of S-octyl GM_3 derivative* **46** *and S-octyl GM_2 derivative* **47**.

4.4.2 In vivo evaluation of the immune response generated by ganglioside-tetanus toxoid conjugates

4.4.2.1 Immunization

The ability of glycoconjugates **34** and **35** to raise a carbohydrate-specific antibody response was evaluated *in vivo*. Groups of five Balb/C mice were immunized with GM_3-tetanus toxoid conjugate **34** and GM_2-tetanus toxoid conjugate **35** using Protocol 1 as discussed in the experimental procedures. A first injection of the glycoconjugates in PBS (50 μg in 100 μL) was administered intraperitoneally along with Freund's complete adjuvant (100 μL). Two subsequent injections (50 μg in 100 μL PBS) with Freund's incomplete adjuvant (100 μL) were made subcutaneously on days 21 and 28 in order to augment the response. Ten days after the last injection, the sera was collected for determination of antibody titer and specificity.

4.4.2.2 Determination of antibody titer for mice immunized with GM_3-TT conjugate

The antibody titer was determined by enzyme-linked immunosorbent assay (ELISA) (Figure 4.1). In this assay, glycoconjugates consisting of different ganglioside fragments linked to bovine serum albumin were immobilized on microtiter plates. Upon incubation of the immune sera, antibodies specific for the immobilized antigen will bind to the immobilized sugar while unbound antibodies will be washed away. The specific binding of antibody is detected *via* an enzyme-linked secondary antibody. Upon addition of the enzyme substrate, a yellow color develops, which is proportional to the amount of bound antibody. The antibody titer is defined as the serum dilution at which the optical density reading is below the background level. In this study the background level was generally below 0.2.

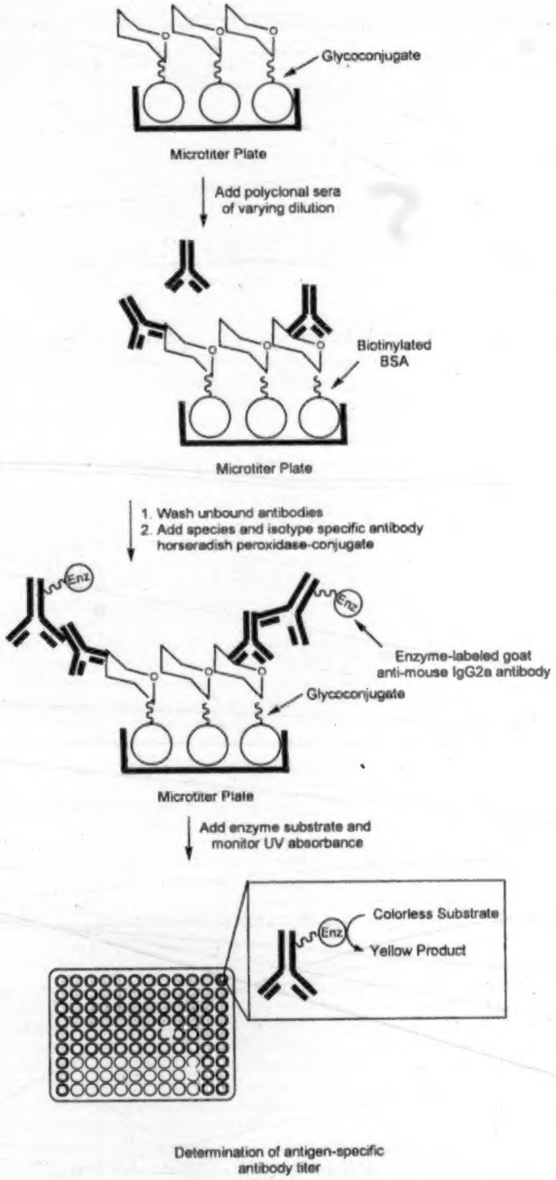

Glycoconjugate
Microtiter Plate
Add polyclonal sera of varying dilution
Biotinylated BSA
Microtiter Plate
1. Wash unbound antibodies
2. Add species and isotype specific antibody horseradish peroxidase-conjugate
Enz
Enz
Enzyme-labeled goat anti-mouse IgG2a antibody
Glycoconjugate
Microtiter Plate
Add enzyme substrate and monitor UV absorbance
Colorless Substrate
Enz
Yellow Product
Determination of antigen-specific antibody titer

Figure 4.1 *ELISA protocol for the determination of antibody titer.*

Preliminary experiments indicated that mice immunized with either GM_3-TT conjugate **34** or GM_2-TT conjugate **35** had a high titer of glycolipid specific antibodies. Mice immunized with GM_3-TT conjugate **34** responded with an average IgM titer of 7×10^3 and an average IgG titer of 3×10^4 against the squarate linked conjugate **40**. (Figure 4.2). In general, the IgM titers were lower than the IgG ones indicating an antigen-induced isotype switch. In order to ascertain that the linker of the immunizing conjugate was not immunogenic itself, the immune sera was screened against GM_3-BSA conjugates **36** and **40** as immobilized antigens. Furthermore, mice immunized with GM_3-TT conjugate **34** developed IgG antibodies that recognized either conjugate to similar degrees (data not shown), suggesting not only that the ganglioside produced an antigen specific immune response, but also that generation of adipate specific antibodies was minimal.

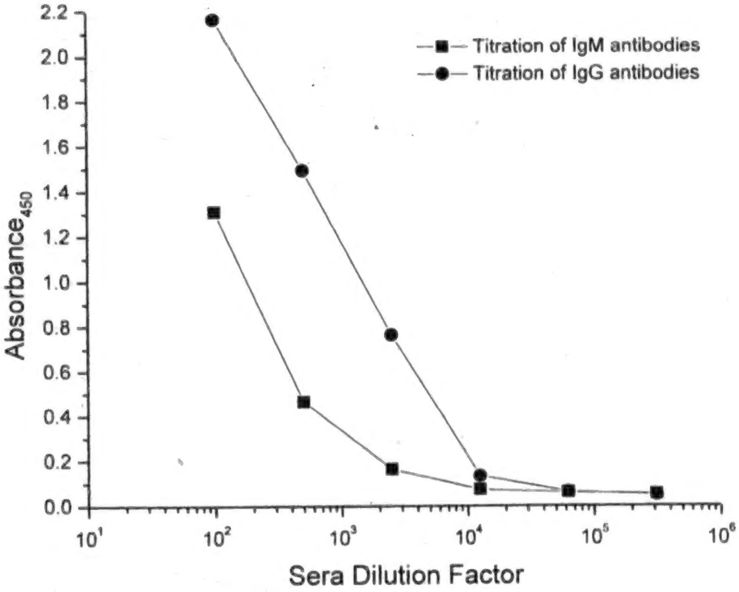


Figure 4.2 *Binding of IgM (square) and IgG (circle) antibody from immune sera obtained from mice immunized with GM_3-TT conjugate* **34** *to GM_3-BSA conjugate* **40**. *Each curve represents the average response of three mice.*

To better understand the immune response to the GM_3-TT antigen and to verify the importance of the terminal sialic acid fragment in antibody recognition, the immune sera was screened against three immobilized antigens incorporating different fragments of the trisaccharide: GM_3 **40**, lactose **42**, glucose **43**, and GM_4 **44** for reactivity with IgG antibodies (Figure 4.3). The difference in IgG antibody titers between the sialylated immobilized antigens (GM_3 and GM_4) and their non-sialylated counterparts (lactose and glucose) indicated that the terminal sialic acid residue is important for antibody recognition. It is noteworthy to mention that the increased reactivity of the immune sera observed against glucose can be explained by the substantially higher substitution of the protein.

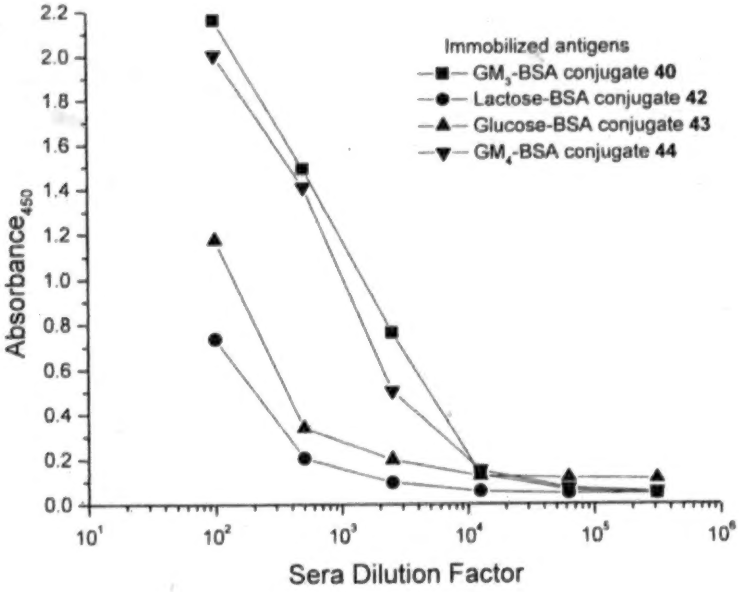


Figure 4.3 *Binding of IgG from immune sera against GM_3-TT conjugate* **34** *to BSA glycoconjugates* **40** *(GM_3 square),* **42** *(lactose, circle),* **43** *(glucose, triangle), and* **44** *(GM_4, down triangle). Each curve represents the average response of three mice immunized with glycoconjugate* **34**.

4.4.2.3 Analysis of immune sera obtained from mice immunized with GM_3-TT conjugate 34 by competitive ELISA

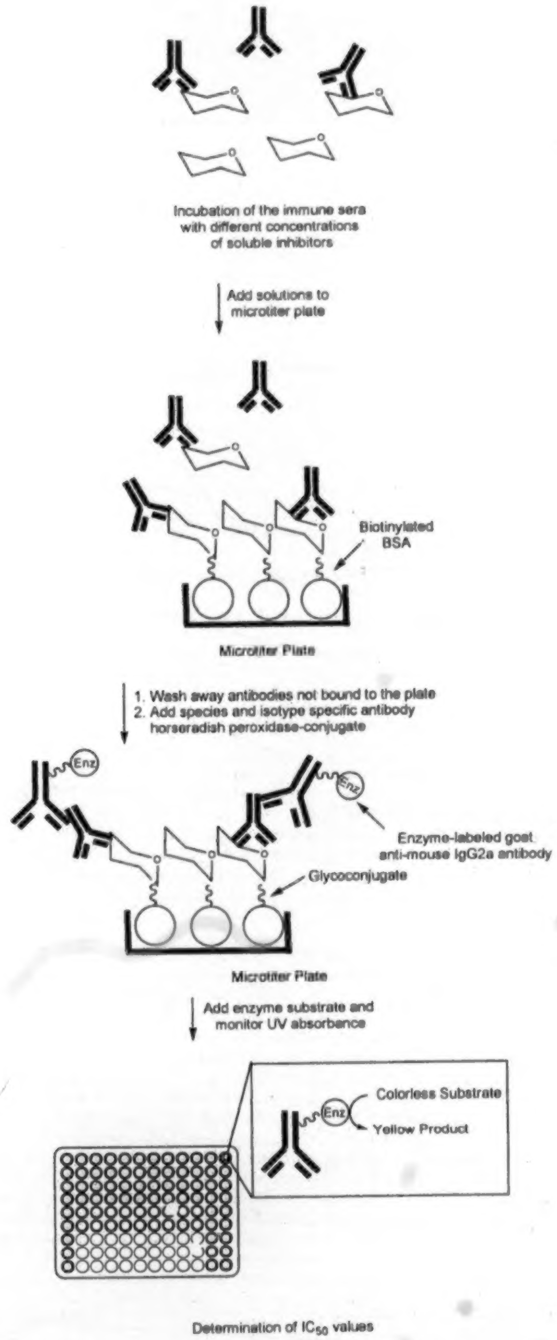

Incubation of the immune sera with different concentrations of soluble inhibitors
Add solutions to microtiter plate
Biotinylated BSA
Microtiter Plate
1. Wash away antibodies not bound to the plate
2. Add species and isotype specific antibody horseradish peroxidase-conjugate
Enz
Enzyme-labeled goat anti-mouse IgG2a antibody
Glycoconjugate
Microtiter Plate
Add enzyme substrate and monitor UV absorbance
Colorless Substrate
Yellow Product
Determination of IC_{50} values

Figure 4.4 *Protocol for determination of* IC_{50} *values.*

In order to establish that the antibodies recognize sialoside-containing epitopes and not just the internal segment of the oligosaccharide hapten, inhibition of antibody binding to glycoconjugate coated microtiter plates was performed in a competitive ELISA experiment. In the competitive ELISA assay, the immune serum is first incubated with different concentrations of a soluble oligosaccharide (the inhibitor). After a certain period of time, the solutions are added to a microtiter plate coated with an immobilized antigen. The unbound antibodies are washed away and binding is detected *via* an enzyme-labeled secondary antibody (Figure 4.4). This assay showed that antibody binding had a strict requirement for the terminal sialic acid residue since GM_3 derivative **30** was at least a 1,000-fold more effective inhibitor than the corresponding lactosyl derivative (IC_{50} of 0.39 μM vs >1000 μM, data not shown). It was also revealed that the ceramide portion of the molecule had an effect on antibody binding, since substitution of the truncated sphingosine aglycon by a *S*-octyl analogue resulted in a 100-fold decrease in IC_{50} values (Figure 4.5).

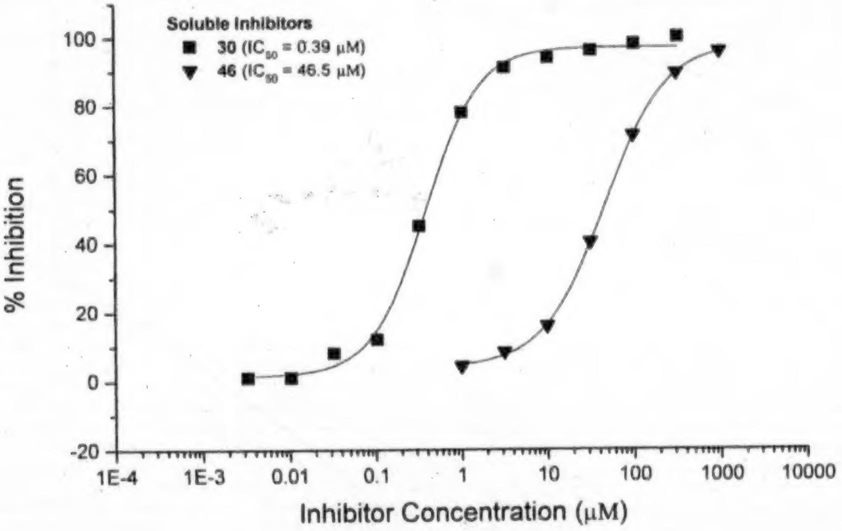


Figure 4.5 *Competitive ELISA assay for immune sera raised against GM_3-TT conjugate* **34**. *Synthetic oligosaccharides* **30** *(square) and* **46** *(down triangle) inhibit binding of IgG to immobilized GM_3-BSA antigen* **40**. *IC_{50} values were determined to be 0.39 μM for* **30** *and 46.5 μM for* **46**.

4.4.2.4 Determination of antibody titer for mice immunized with GM_2-TT conjugate 35

Similar results were observed for mice immunized with GM_2-TT conjugate **35**. The mice responded to immunization with an average IgM titer of 1×10^4 and an average IgG titer of 8×10^4 against the immobilized GM_2-BSA conjugate **41** (Figure 4.6). Again, the higher IgG titer corresponds to isotype switching from IgM antibodies to higher-affinity IgG antibodies showing involvement of a T_H cell response. The antibody titer of mice immunized with the GM_2 glycoconjugate was further analyzed and determined for five different immobilized antigens: GM_2-BSA **41**, GM_3-BSA **40**, GM_4-BSA **44**, lactose-BSA **42**, and glucose-BSA **43**. As shown in Figure 4.7, the difference in titers between the immobilized antigens suggested that both the *N*-acetylgalactosamine and sialic residues might be important for antibody recognition as in absence of these residues the antibody titer decreases several fold.

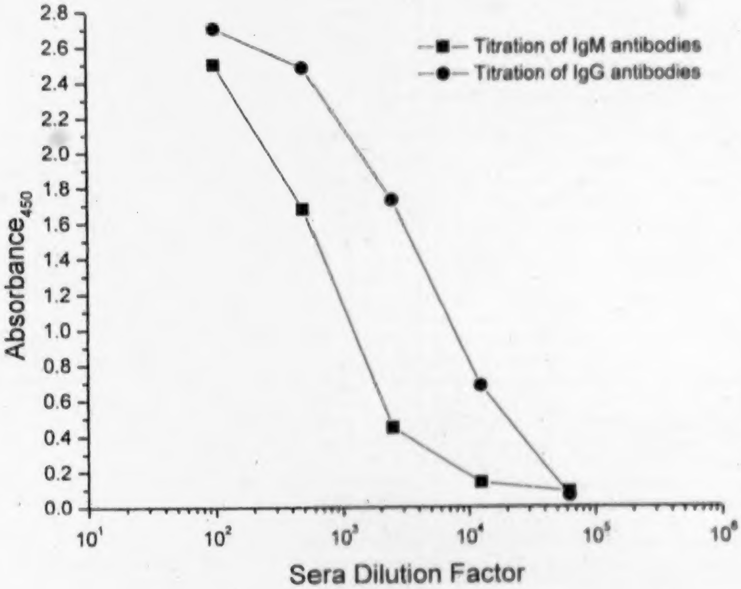


Figure 4.6 *Binding of IgM (square) and IgG (circle) antibody from immune sera obtained from mice immunized with GM_2-TT conjugate* **35** *to GM_2-BSA conjugate* **41**. *Each curve represents the average response of three mice immunized with glycoconjugate* **35**.

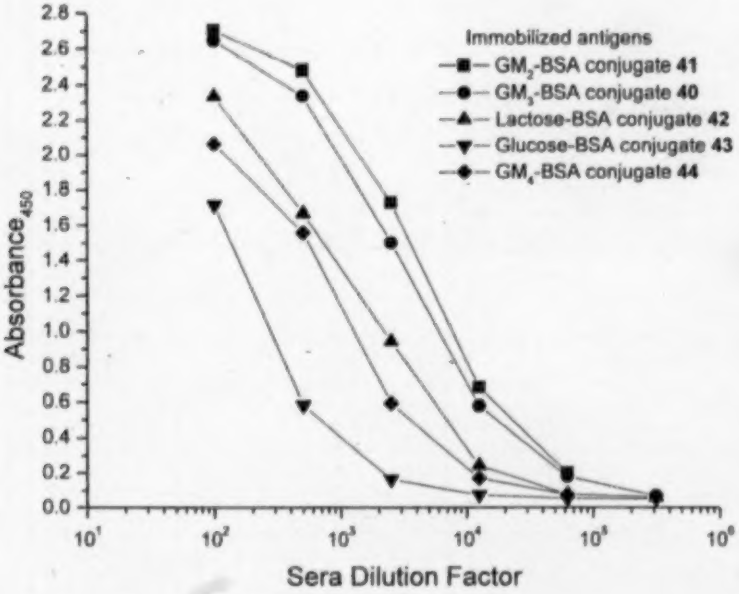


Figure 4.7 *Binding of IgG from immune sera against GM_2-TT conjugate* **35** *to BSA glycoconjugates* **41** *(GM_2, square),* **40** *(GM_3, circle),* **42** *(lactose, triangle),* **43** *(glucose, down triangle) and* **44** *(GM_4, diamond). Each curve represents the average response of three mice immunized with glycoconjugate* **35**.

4.4.2.5 Analysis of immune sera obtained from mice immunized with GM_2-TT conjugate 35 by competitive ELISA

Further study of the binding epitope by competitive ELISA experiments confirmed this statement. The immune serum of the highest responsive mouse was screened against four soluble analogues: the GM_2-tetrasaccharide **31** , the GM_3-trisaccharide **30**, and the lactoside containing either a truncated ceramide or *S*-octyl aglycon (Figure 4.8). Comparison of the IC_{50} values for the three different saccharides indicated that the GM_2-

derivative is a more potent inhibitor by approximately 50-fold, revealing the importance of both the *N*-acetylgalactosamine and sialic acid moieties in antibody binding. In a similar way, comparison of the IC_{50} values for the compounds containing the truncated ceramide and for the compounds containing the *S*-aglycon indicated that the ceramide portion of the molecule is also recognized by antibodies, corroborating the results observed in mice immunized with the GM_3 conjugate. Substitution of the ceramide end of the molecule affects antibody binding to a smaller extent than deletion of either the terminal sialic acid or *N*-acetylgalactosamine moiety.

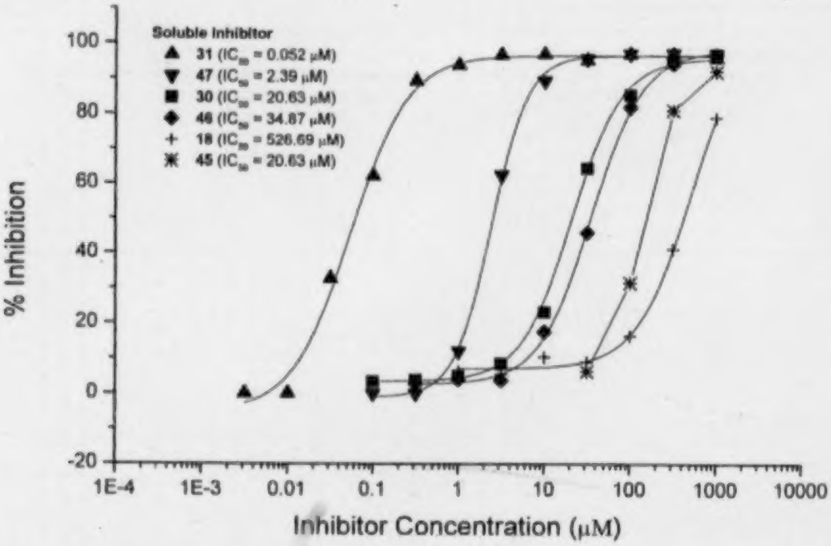


Figure 4.8 *Competitive ELISA assay for immune sera raised against GM_2-TT conjugate* **35**. *Synthetic oligosaccharides* **31** *(triangles),* **47** *(down triangles),* **30** *(square),* **46** *(diamond),* **18** *(cross), and* **45** *(star) inhibit binding of IgG antibodies to immobilized GM_2-BSA antigen **41**. IC_{50} values were determined to be 0.052 μM for* **31** , *2.39 μM for* **47**, *20.63 μM for* **30**, *34.87 μM for* **46**, *20.63 μM for* **18** , *and 526.69 μM for* **45**.

4.4.3 Analysis of the effect of the aglycon on ganglioside immunogenicity

4.4.3.1 Preparation of ganglioside conjugates incorporating different lipid fragments

Immunochemical data obtained in this study indicated that ceramide elements are recognized by antibodies induced by glycoconjugates **34** and **35**. This suggests that it can be important to include at least the stereogenic centres of the ceramide portion of gangliosides in conjugate vaccine design. In order to assess the extent of the importance of the ceramide in the vaccine construct and its point of attachment to the carrier protein, three different GM_3-TT conjugates incorporating different segments of the lipid were prepared. As shown in Figure 4.9, a GM_3 derivative incorporating an amino-terminated lipid aglycon **48**, another one bearing a truncated ceramide **49**, and lyso-GM_3 **50** were conjugated to tetanus toxoid *via* the highlighted amino group.[5] A first attempt was made to conjugate these oligosaccharides to tetanus toxoid *via* the *p*-nitrophenyl adipate diester previously used. However, the increased lipophilicity of the antigen was incompatible with this conjugation method and very low incorporation levels were obtained (<1.0) even with the help of extensive sonication.[6] A different conjugation strategy was therefore needed. Tetanus toxoid was first derivatized with *N*-succinimidyl bromoacetate in PBS buffer, pH 7.2 under controlled conditions to obtain approximately 20 bromide residues. After removal of excess reagent by size-exclusion chromatography, mass spectrometric analysis revealed that 21 bromide residues were added to the protein. (Scheme 4.4). Having in hand the bromoacetylated protein, a thiol group needed to be incorporated in each of the oligosaccharide antigens. As described in Scheme 4.4, the three GM_3-derivatives **48**, **49**, **50** were treated with *N*-succinimidyl acetylthioacetate in the presence of 4-*N*,*N*-dimethylaminopyridine to yield precursors bearing a protected thiol. This conversion afforded the targeted intermediate in 83% yield for **52**, 88% yield for **53** and 94% for **54**. These compounds were subsequently treated with a deacetylation solution consisting of hydroxylamine in PBS, pH 7.2, complemented with a small amount of EDTA to prevent oxidation of the deprotected thiol. The thiol bearing antigens were then incubated in the presence of bromoacetylated tetanus toxoid to effect conjugation to the carrier protein. Purification by size-exclusion chromatography afforded conjugates **58**, **59**, and **60** with hapten incorporation levels of 4, 6, and 4 respectively (Scheme 4.4).

---

[5] The amino-functionalized oligosaccharides were kindly provided by Dr. Ping Zang and Dr. Chang-Chun Ling.

[6] Personal communication with Dr. Ping Zang and Dr. Chang-Chun Ling.

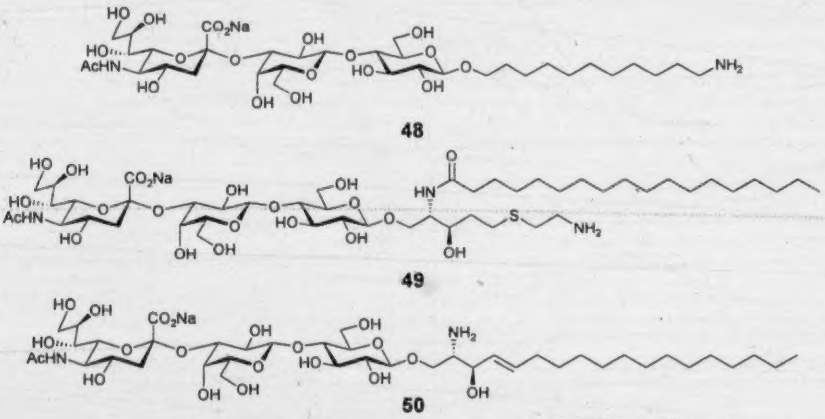
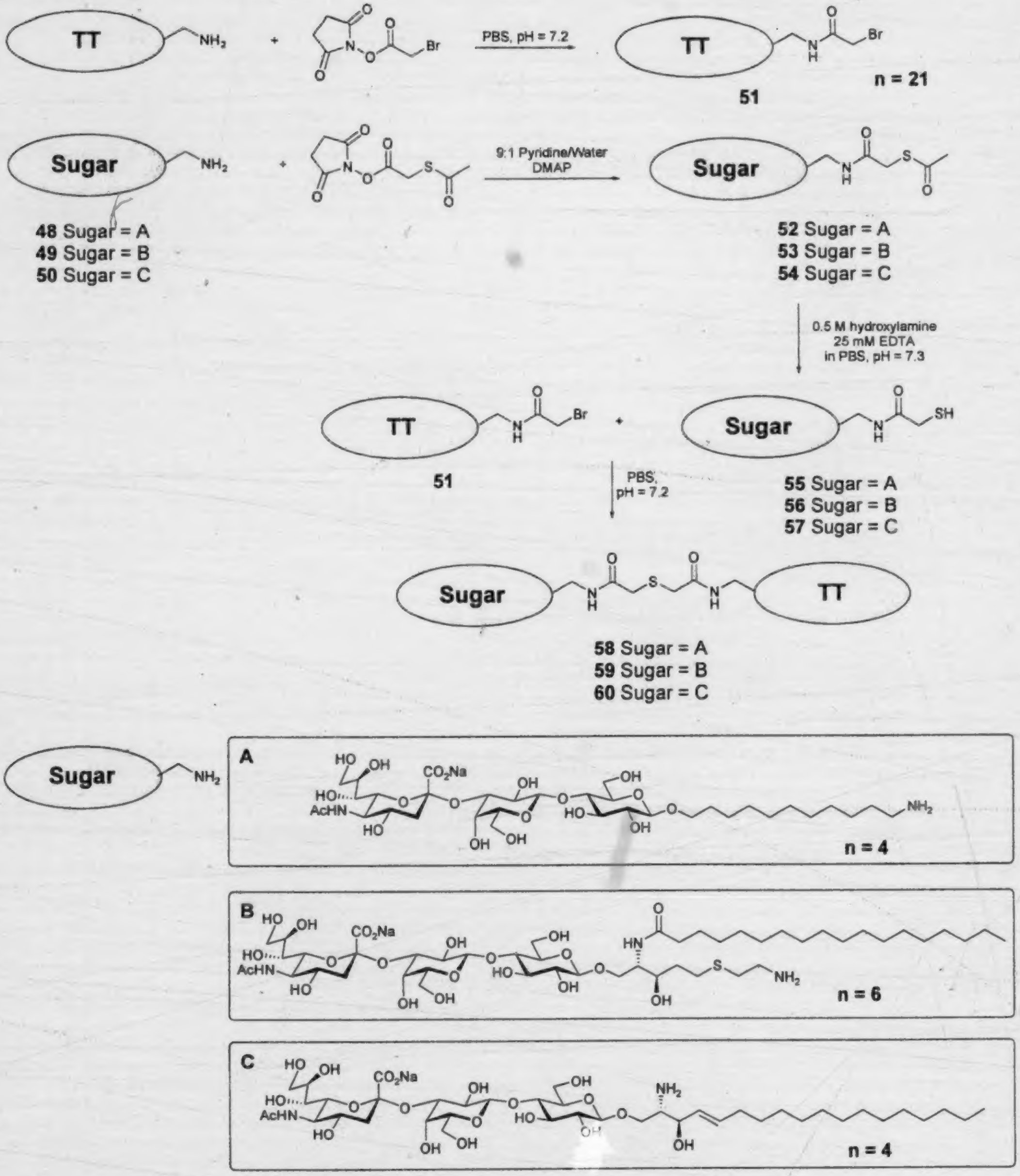
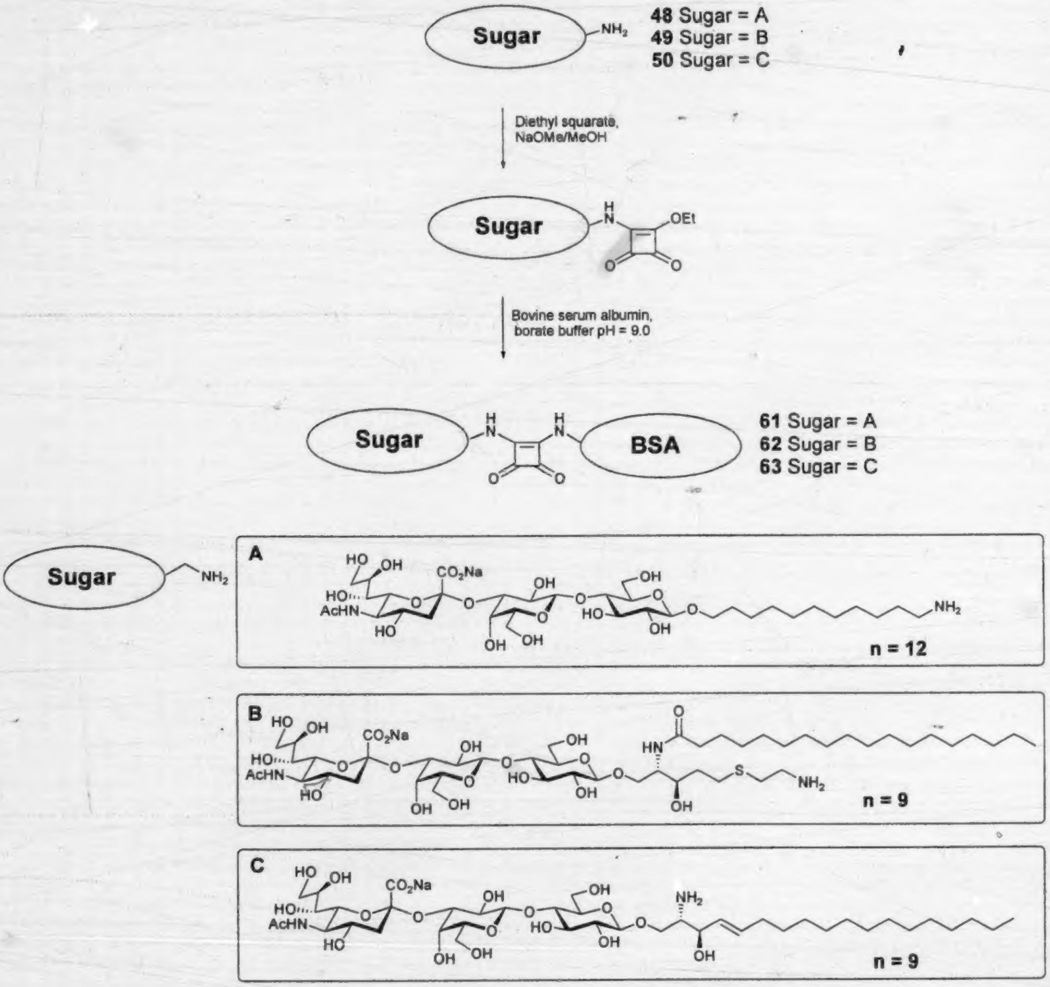


Figure 4.9 Structure of the glycolipid haptens used in this study.

In order to evaluate the antibody titer generated from immunization with glycoconjugates **58**, **59**, and **60**, a set of conjugates incorporating a heterologous linker and carrier protein was required. Thus, bovine serum albumin conjugates, each incorporating a glycolipid hapten of a different nature, were prepared using diethyl squarate as coupling reagent (Scheme 4.5). Glycolipids **48**, **49**, and **50** were first activated by reaction with diethyl squarate in methanol at pH 9.0. After evaporation of the solvent and resuspension of the residue into borate buffer, pH = 9.0, bovine serum albumin was added to the solution to effect conjugation. Extensive dialysis against milliQ filtered water followed by lyophilization afforded BSA conjugates **61**, **62**, and **63** with incorporation levels of 12, 9, and 9 respectively.


TT
NH2
PBS, pH = 7.2
Br
n = 21
51
Sugar
9:1 Pyridine/Water
DMAP
48 Sugar = A
49 Sugar = B
50 Sugar = C
52 Sugar = A
53 Sugar = B
54 Sugar = C
0.5 M hydroxylamine
25 mM EDTA
in PBS, pH = 7.3
SH
55 Sugar = A
56 Sugar = B
57 Sugar = C
PBS,
pH = 7.2
58 Sugar = A
59 Sugar = B
60 Sugar = C
A
B
C
CO2Na
AcHN
n = 4
n = 6
n = 4

Scheme 4.4 Preparation of the GM_3-TT conjugates **58**, **59**, and **60**.


Sugar
NH₂
48 Sugar = A
49 Sugar = B
50 Sugar = C
Diethyl squarate, NaOMe/MeOH
OEt
Bovine serum albumin, borate buffer pH = 9.0
BSA
61 Sugar = A
62 Sugar = B
63 Sugar = C
A
n = 12
B
n = 9
C
n = 9

Scheme 4.5 *Preparation of GM$_3$-BSA conjugates* **61**, **62**, *and* **63**.

4.4.3.2 Determination of antibody titers for mice immunized with ganglioside conjugates incorporating different lipid fragments

Gangliosides-TT conjugates **58**, **59**, and **60** were evaluated *in vivo* using the same immunization protocol described in Section 4.4.2.1. The antibody titer resulting from immunization with each of the glycoconjugate was determined by an ELISA assay described in Section 4.4.2.2 in which the corresponding glycolipid was immobilized in the microtiter plate.

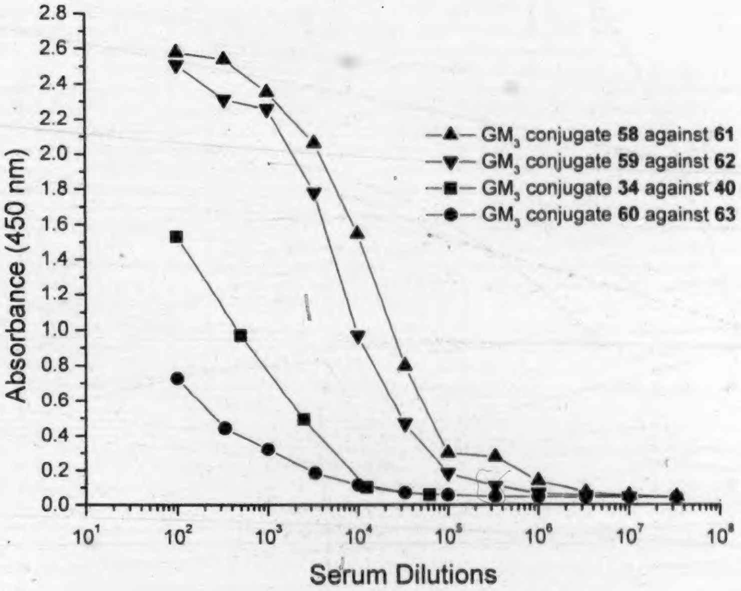


Figure 4.10 *Binding of IgG from immune sera against* GM_3*-TT conjugates* **58**, **59**, **34**, *and* **60** *to BSA glycoconjugates* **61** *(triangle),* **62** *(down triangle),* **40** *(square), and* **63** *(circle) respectively. Each curve represents the average response of five mice immunized with the respective glycoconjugates.*

It was found that the nature of the aglycon of the hapten and its site of conjugation have a significant effect on the immunogenicity of the glycoconjugate. As shown in Figure 4.10, mice immunized with glycoconjugates **58** and **59** responded with an antibody titer of 1×10^6 and 3×10^6 respectively, which are at least one order of magnitude higher than the ones obtained for mice immunized with glycoconjugates **34** (3×10^4) and **60** (1×10^4). These titers represent the relative concentration of glycolipid-specific antibodies as the use of squarate-linked BSA conjugates precludes the detection of antibodies against the linker and the carrier protein. In all cases, nonspecific binding was very low. It therefore appears that the formation of glycoconjugates *via* the fatty-acid chain of the ceramide aglycon have deleterious effects on antibody formation resulting in a lower immune response against the glycolipid. Furthermore, the glycolipid hapten incorporating a simple aliphatic chain as opposed to elements of the ceramide moiety was superior at generating a glycolipid-specific immune response. This result was somewhat to be expected as this hapten has the least resemblance to the natural glycolipid.

4.4.3.3 Evaluation of antibody binding on melanoma cells

In the context of tumor immunotherapy, not only must the vaccine construct be able to generate a high titer of antibodies specific for the hapten, the antibodies formed must also recognize the target antigen in its natural context. Since the sphingosine portion of the ganglioside is relatively polar with its amino and hydroxyl groups, it is reasonable to assume that some of these elements protrude from the cell's plasma membrane and can serve as recognition elements for antibodies. It still remains an open question as to whether or not the antibodies generated from glycoconjugates containing portions of the ceramide moiety as an algycon bind better to the targeted ganglioside in a cellular context than the ones generated from glycoconjugates containing a simple aliphatic chain. The B16-F10 melanoma cell line is a good candidate to address this question in a cellular ELISA assay as it is known to express very high levels of ganglioside GM_3.[7] The assay is very similar to the ELISA assay described in Section 4.4.2.2 except suspensions of cells at various dilutions are added to the microtiter plate instead of ganglioside-BSA conjugates. This process was, however, unsuccessful as the adhesion of the cells appeared to be disrupted. Fixation of the cells with formaldehyde did not yield more meaningful data. No binding was detected in any of the wells including the positive control consisting of the anti-GM_3 monoclonal antibody GMR-6. It was thus hypothesized that the fixation procedure could have disrupted the cell-surface antigen, thereby preventing binding of the antibodies. We then turned our attention to flow cytometry to detect binding of the immune sera to the melanoma cells. In this assay, cultured cells were incubated for an extended period with various dilutions of immune sera or the GMR-6 monoclonal antibody as a positive control. After removal of the unbound species and washing, the cells are incubated with a fluorescently labeled goat anti-mouse secondary antibody for a few hours. After washing, the cells were analyzed by flow cytometry to detect fluorescence and therefore binding to individual cells. Unfortunately, no conclusive results were obtained as no significant binding was detected even with the positive control. It would be of interest to test the cell line, fluorescently labeled secondary antibody, and the anti-GM_3 monoclonal antibody in independent assays to rule out possible problems with these reagents. Of the three assays presented above, the flow cytometry based approach appeared to be the simplest and the less labour-intensive one and seem worth pursuing further. Literature searches revealed that the time of incubation and the concentration of the different antibodies involved greatly varied from one case to the other. Therefore varying the different parameters of the experiment over a wider range may prove useful in finding working conditions.

---

[7] The author would like to thank Dr. Mavanur Suresh for kindly providing us with the B16-F10 cell line.

4.5 Conclusions

One of the main goals of tumor immunotherapy is to break the immune system tolerance to tumor associated antigens. In other words, to generate a high concentration of antigen-specific antibodies that have a high binding affinity for the target. This task is particularly challenging as most of these antigens are complex oligosaccharides naturally found in low concentration under healthy conditions and act on their own as T_H-cell independent antigens. We have shown that conjugation of different gangliosides to tetanus toxoid generated high titers of hapten-specific IgG antibodies ranging from 10^4 to 10^6 in a conservative immunization protocol. These results stands beside other studies using KLH conjugates and confirm the utility of tetanus toxoid in tumor immunotherapy as a potent carrier that is easy to manipulate. Immunization with GM_3- and GM_2-tetanus toxoid conjugates incorporating a truncated ceramide algycon demonstrated that GM_2 was a slightly superior immunogen. In both cases, titers for IgG antibodies were higher than those for IgM antibodies. The preponderance of the higher affinity IgG subtype is indicative of a desired antibody class switch and activation of T_H cells. The immunochemical data resulting from this study demonstrates that the binding site of the antibodies generated by the GM_3 immunogen spans not only the sialyl moiety but also some elements of the ceramide aglycon. Similar results were observed for mice immunized with the GM_2 conjugate. This time the antibodies recognized both the sialic acid and *N*-acetylgalactosamine residues as well as a portion of the ceramide moiety.

Further studies showed that the nature of the lipid moiety of the hapten and its site of conjugation affect its immunogenicity. It was found that a GM_3 glycoconjugate incorporating a simple aliphatic chain as aglycon was superior at inducing an oligosaccharide-specific response with an antibody titer of 3×10^6. Conjugation *via* the fatty acid chain of the ceramide appeared to have some deleterious effect the immunogenicity as these conjugates induced an antibody response at least two order of magnitude lower.

The generation of high titers of antigen-specific antibodies is not the only important factor to consider in the development of a vaccine. In the context of tumor immunotherapy, it is also of equal importance to generate antibodies that recognize the target antigen in its setting on the cancer cell. We have shown that the binding site of antibodies generated against GM_3 and GM_2 conjugates span beyond the oligosaccharide portion of the glycolipid. As the natural gangliosides bear a lipid that have a relatively polar sphingosine head, it is thought that this part of the ganglioside protrudes from the cell membrane and could thus be available for antibody binding. Even though the GM_3 glycoconjugate incorporating a simple aliphatic aglycon generated the most elevated oligosaccharide-specific titers, this conjugate might not be the vaccine of choice for therapeutic applications as it does not include part of the ganglioside recognition elements, namely the sphingosine moiety. Cellular assays evaluating the binding of the different immune sera to cancer cells overexpressing the tumor associated antigen is a more relevant basis for determining the therapeutic relevance of the immunogen. As meaningful results were not obtained from attempts to assess antibody binding to cancer cells, it is still an open question as to whether or not the ceramide moiety or at least some elements of it should be included in the design of ganglioside-based conjugate vaccines.

Chapter 5

Targeting Glycoconjugates to Dendritic Cells

Encouraged by the immune responses obtained from the immunization of mice with the different ganglioside constructs presented in Chapter 4, we were looking to further enhance the immunogenicity of these glycoconjugates. In collaboration with Mr. Welson Wang in the laboratory of Dr. Suresh, a new vaccination strategy was investigated whereby the antigens are targeted to dendritic cells in order to increase their capture and presentation to the immune system and potentially improve their immunogenicity. This chapter describes the derivatization of different ganglioside constructs for delivery to dendritic cells using a bispecific fusion protein and evaluation of the immune response against the ganglioside portion of the molecules.

5.1 Role of dendritic cells in the immune system

Dendritic cells play a central role in adaptive immunity by controlling the function of B and T lymphocytes.[246-250] As discussed in Section 1.4, T lymphocytes transduce antigen recognition into effector mechanisms. Once activated, T lymphocytes can complete the immune response by interacting with other cells such as B cells for antibody production, macrophages for cytokine release and target cells for lysis. In order for T lymphocytes to recognize antigens, they must be processed and presented on the surface of specialized antigen presenting cells (APCs) in complex with a major histocompatibility complex (MHC) molecule. Depending on its origin, the antigen can be presented on a MHC class I or MHC class II molecule affecting the sub-population of T lymphocytes activated. Intracellular antigens presented in complex with an MHC class I molecule activate CD8$^+$ cytotoxic T (T_c) lymphocytes leading to lysis of infected or tumor cells. In contrast, extracellular antigens presented in complex with an MHC class II molecule activate CD4$^+$ helper T (T_H) lymphocytes leading to the release of cytokines which stimulate B cell differentiation, proliferation, and antibody production.

Different cell types, including dendritic cells, macrophages and B lymphocytes can act as antigen presenting cells. However, dendritic cells are by far the most potent antigen presenting cell. Located in most tissues, dendritic cells circulate in the periphery where they capture and process antigens. Upon uptake of an antigen, dendritic cells upregulate the expression of MHC and co-stimulatory molecules at their surface and migrate to the lymphoid organs forming clusters with antigen-specific T lymphocytes. These clusters create a microenvironment in which the mature dendritic cells can interact with T lymphocytes and stimulate their activation *via* binding of adhesion and signaling molecules to cell-surface receptors determining the fate of the immune response. Depending on the nature of MHC-restriction and the type of cytokines released, dendritic cells can stimulate either cellular or humoral immunity. Given their central role in controlling immune functions, dendritic cells constitute a promising target for many clinical applications including vaccine development, treatment of autoimmune disease and immunotherapy.

5.2 Dendritic cells and cancer immunotherapy

Antigen presentation is a critical feature of any immunization strategy and one way to enhance the response against tumor antigens is to manipulate the way in which the antigen is presented. The modulation of dendritic cell functions have been applied in two distinct ways to treat cancer.[251,252] In one approach, dendritic cells generated *ex-vivo* are pulsed with a pre-determined tumor antigen or fused with an autologous or allogenic tumor cell and re-introduced into the bloodstream of the patient.[253,254] This approach takes advantage of the ability of dendritic cells to migrate to the T-cell area of the lymphoid organs and induce a strong T-cell response. Although several phase I clinical studies using this methodology have been undertaken, only a few showed tumor regression and none have yet shown an improved patient survival rate.[255,256]

Another promising approach involves directly targeting inactivated tumor cells or antigens to dendritic cells with anti-DC monoclonal antibodies. Typically, the antigen is delivered to dendritic cells by a monoclonal antibody specific for a cell-surface receptor along with a stimuli to control their maturation.[252] Several receptors at the surface of dendritic cells have been explored as potential targets including, DC-SIGN/CD209, MMR/CD206/MRC-1, DCIR/CLEC4A,[257] DEC-205/CD205, Lox-1/OLR1,[258] toxin receptors, and CD40. Of particular interest is the DEC-205 receptor, an integral membrane protein, homologous to the macrophage mannose receptor (MMR) which mediates endocytosis.[259] Dendritic cells expressing DEC-205 are widely distributed throughout the body and are present as both immature and mature populations.[260,261] DEC-205 is expressed at particularly high levels at the surface of dendritic cells within the T cell area of lymphoid tissues where the effector mechanisms of the immune response are initiated.[260,262,263] DEC-205 traffics in a distinct way through dendritic cells and greatly enhances antigen presentation relative to other endocytic receptors as they circulate through the late endosomal/lysosomal vacuole of maturing dendritic cells, a compartment enriched with MHC molecules and proteinases which favor antigen processing and presentation.[264] It has been shown that targeting an antigen to dendritic cells *via* the DEC-205 receptor delivers the antigen onto both MHC I and MHC II and increases the presentation efficiency by 100-1000-fold relative to non-targeted antigens.[265,266] Despite significant advances, dendritic cell-based immunotherapy against cancer is still an underdeveloped area. Yet this approach can exploit the patient's immune response to cancer to produce more specific, less toxic, broader and longer lasting therapies.

5.3 Enhancing the immune response to gangliosides using a bispecific monoclonal antibody

In diseased states such as cancer and infection, dendritic cells can exhibit impaired function including a combination of inefficient migration and impaired antigen uptake and processing. Targeting strategies with co-stimulation have the potential to overcome some aspect of migratory deficiencies and facilitate the uptake process. Antibody-mediated targeting of an antigen to dendritic cells *via* the DEC-205 receptor was shown to greatly increase immunity.[265,267] It was thus envisaged that the DEC-205 mediated delivery of a ganglioside conjugate to dendritic cells could significantly enhance the immune response to the glycoconjugate. Generation of an antibody-mediated antigen targeting system is a complex and time consuming process, which requires either encapsulation of the antigen,[268] chemical cross-linking of the antigen with the antibody[263,269] or construction of a new genetically-engineered hybrid antibody.[270] This process requires considerable effort as the system needs to be adapted for each antigen. The laboratory of Dr. Suresh in the Pharmacy Department at the University of Alberta has developed a universal dendritic cell targeting vehicle that can conveniently deliver any antigen to dendritic cells.[271] This targeting vehicle consists of a bispecific monoclonal antibody bearing two distinct paratopes-one specific for the dendritic cell receptor DEC-205 and one specific for biotin. Immunization of mice with this ovalbumin along with this bifunctional antibody and a dendritic cell activator was shown to give high titers of ovalbumin-specific antibodies.[271] However, the use of bispecific monoclonal antibodies in clinical applications presents several limitations. The F_c region of the antibodies can cause undesirable immune responses and the pharmacokinetic profile of these antibodies is less than ideal. New strategies to redesign these bifunctional constructs for clinical applications have shown promising results. Notably, bispecific fusion protein constructs, which lack F_c domains have reduced side effects. The group of Dr. Suresh have developed a bispecific fusion protein construct to target a biotinylated antigen to dendritic cells. This bispecific construct incorporate a single chain antibody (scFv) against the receptor DEC-205 and a truncated streptavidin. Our strategy consisted in using this bispecific fusion protein along with a co-stimulatory component to target biotinylated ganglioside conjugates to dendritic cells in order to increase their capture and presentation to T_H cells and enhance their immunogenicity (Figure 5.1).

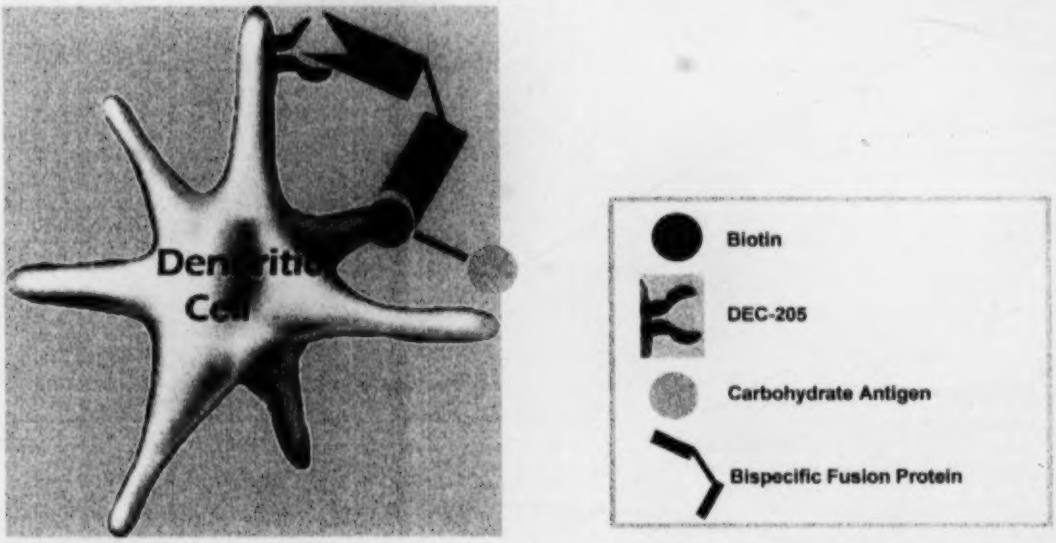


Figure 5.1 *Targeting a glycoconjugate vaccine to dendritic cells using a bispecific fusion protein construct.*

5.4 Results and discussion

5.4.1 Development of a bispecific fusion protein[8]

Bispecific fusion proteins are unique heterobifunctional macromolecules which bear two different binding specificities within a single molecule.[272,273] These recombinant molecules are generated by a hybridoma cloning method. The hybridoma cloning method involves culturing of the hybridoma, extraction of the total RNA and purification of mRNA using an oligo dT purification system. The purified mRNA is used as a template to synthesize strands of cDNA corresponding to the VH and VL genes. These cDNA sequences are then amplified using VH and VL specific primers for the construction of the scFv antibody. The full length scFv is generated by combining equal amounts of VH and VL PCR products with 15 amino acid linkers and assembled by polymerase chain reaction. Fusion constructs comprising antibodies fragments with proteins or peptides have been generated by recombinant DNA technology. The construction involves cloning of scFv gene at the N-terminus and the CS gene at the C-terminus following by His_5 tag.

---

[8] This work was carried out by Mr. Welson Wang, Department of Pharmacy, University of Alberta.

The scFv gene is usually followed by either pelB or CS leader sequence. A spacer of 5 amino acids (Glycine-Serine-Glycine-Alanine-Alanine or Glycine-Serine-Glycine-Serine-Alanine) is usually placed in between the scFv gene and core steptavidin gene. The fusion protein is found in 3 isoforms: monomeric, dimeric and tetrameric form due to the tendency of the core streptavidin to form oligomers.

A scFv that recognizes the DEC-205 receptor at the surface of dendritic cells was successfully cloned from a rat hybridoma that secretes anti DEC-205 IgG_{2a} monoclonal antibodies (HB290) and fused with a core-streptavidin domain. The bispecific construct was expressed in *E.coli* using a T7 expression system. The monomeric form of the fusion protein was purified by affinity chromatography. The bifunctional activity was demonstrated by ELISA as shown in Figure 5.2.

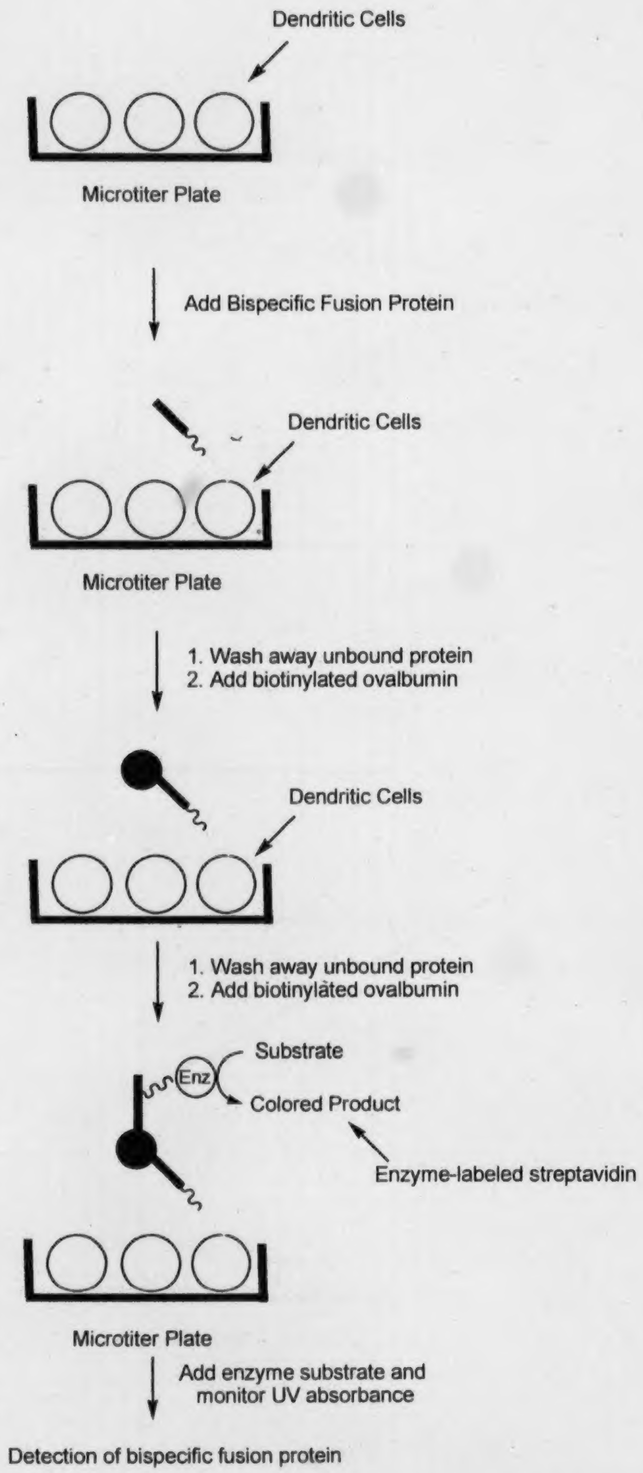
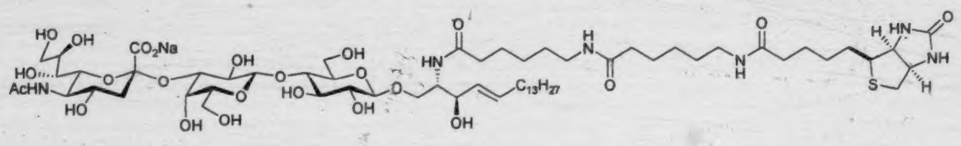

Dendritic Cells
Microtiter Plate
Add Bispecific Fusion Protein
Dendritic Cells
Microtiter Plate
1. Wash away unbound protein
2. Add biotinylated ovalbumin
Dendritic Cells
1. Wash away unbound protein
2. Add biotinylated ovalbumin
Substrate
Enz
Colored Product
Enzyme-labeled streptavidin
Microtiter Plate
Add enzyme substrate and
monitor UV absorbance
Detection of bispecific fusion protein

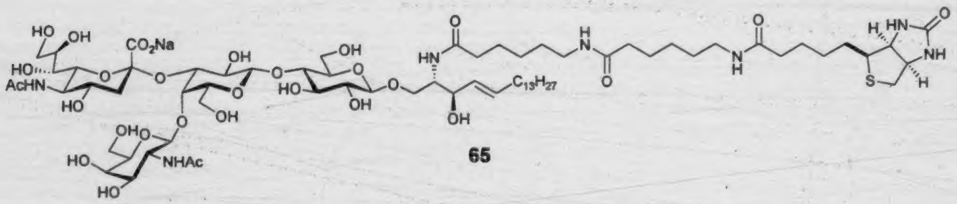
Figure 5.2 *ELISA protocol for detecting bispecific fusion protein.*

5.4.2 Biotinylation of ganglioside antigens

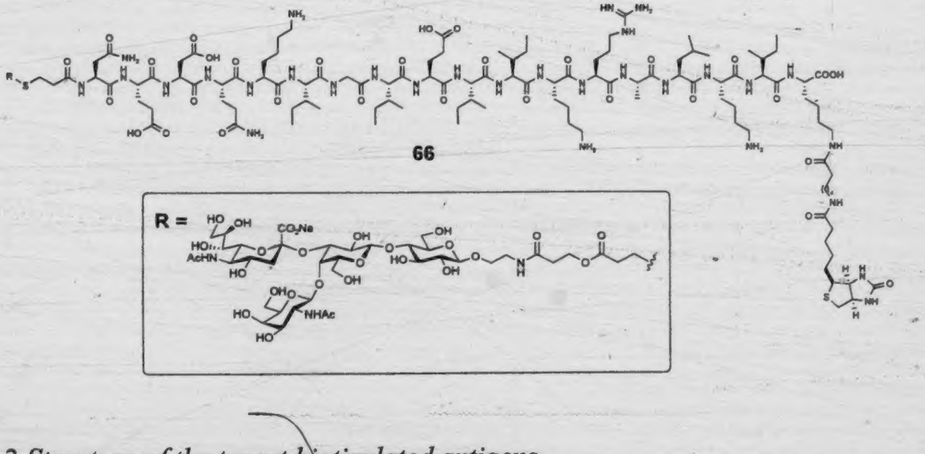
Several studies have shown that proteins or peptides targeted to dendritic cells *via* the DEC-205 receptors have enhanced immunogenicity compared to the untargeted antigen. Furthermore, *in vivo* studies by Wang *et al.* showed that immunization of mice with biotinylated ovalbumin in the presence of a bispecific monoclonal antibody and the co-stimulatory anti-CD40 monoclonal antibody can augment both humoral and cell-mediated responses.[271] We were curious as to whether or not the same effect would be observed for an oligosaccharide hapten administered in conjunction with the bispecific fusion protein. In order to test this hypothesis, biotinylated immunogens were required. Three different immunogens were prepared to test this immunization strategy including biotinylated GM_3 **64** and GM_2 **65** and a biotinylated glycopeptide **66** (Figure 5.3).



64



65

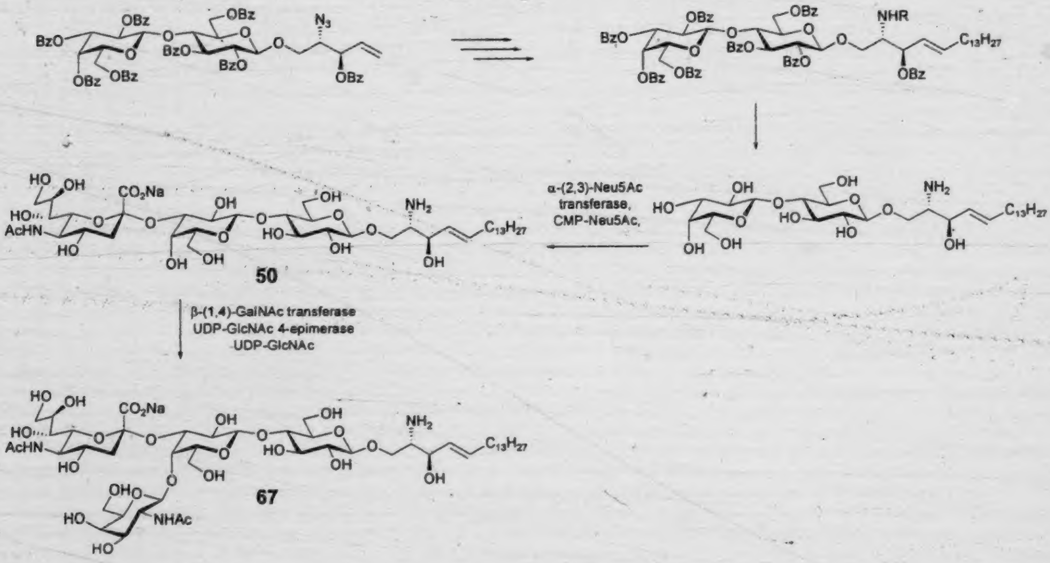


66

Figure 5.3 *Structure of the target biotinylated antigens.*

5.4.2.1 Synthesis of biotinylated GM$_3$- and GM$_2$- glycolipids

Gangliosides GM$_3$ **50** and GM$_2$ **67** incorporating a long olefinic lipid were synthesized as outlined in Scheme 5.1.[9] The lipid portion of truncated lactosyl sphingosine was first extended by olefin cross metathesis using Grubb's second generation catalyst and 1-pentadecene as a coupling partner to afford the target compound with excellent *E*-selectivity. The compound was then debenzoylated and the azide was reduced to afford the corresponding lactoside which was soluble enough in basic aqueous solutions to allow for enzymatic reactions. Subsequent elaboration by enzymatic glycosylation yielded the GM$_3$ and GM$_2$ glycolipids **50** and **67** in excellent yield after purification on normal phase silica gel. The glycolipids were subsequently biotinylated as shown in Scheme 5.2 with biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester affording the derivatives necessary for our immunization study in good yields.

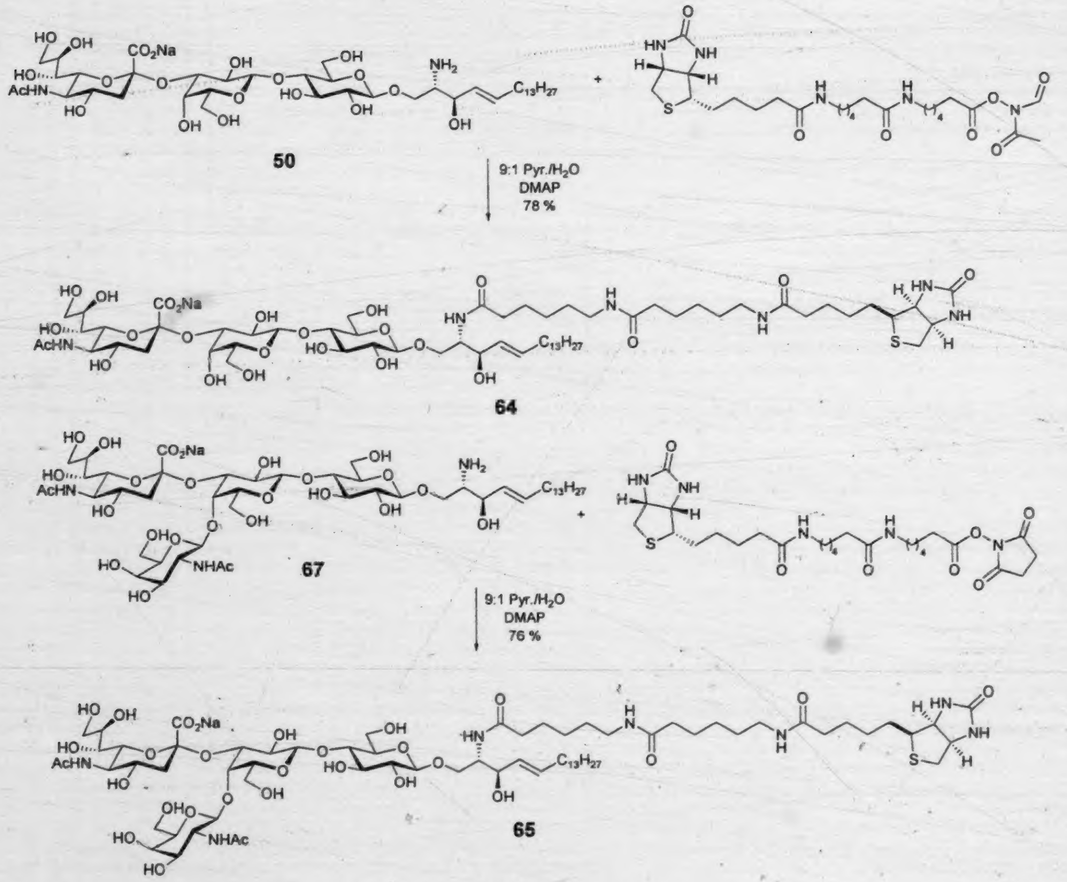


α-(2,3)-Neu5Ac transferase, CMP-Neu5Ac,
β-(1,4)-GalNAc transferase
UDP-GlcNAc 4-epimerase
UDP-GlcNAc
50
67

Scheme 5.1 *Synthesis of GM$_3$* **50** *and GM$_2$* **67** *glycolipids.*

---

[9] The author is grateful to Dr. Ping Zhang and Dr. Chang-Chun Ling for providing glycolipids **50** and **67**.



Scheme 5.2 *Biotinylation of GM_3 and GM_2 glycolipids.*

5.4.3 In vivo evaluation of the immune response generated by the concomitant injection of biotinylated gangliosides and the bispecific fusion protein

The ability of the bifunctional fusion protein to efficiently enhance the immune response against the two biotinylated ganglioside antigens was evaluated *in vivo*. Groups of five Balb/c mice were injected subcutaneously near the inguinal lymph node with different combinations of antigens, fusion protein and co-stimulatory molecules. The immunization protocol is described in detail in Table 5.1. Group 1 constituted the negative control group. The mice of this group were only injected with PBS at different times of the immunization schedule. The second group, Group 2, was the test group. The mice of this group were immunized at day 0 with a combination of biotinylated GM_3 **64**

(1 μg), biotinylated GM_2 **65** (1 μg), the bispecific fusion protein (20 μg) and a co-stimulatory component, the anti-CD40 monoclonal antibody (25 μg) in saline. The immune response was boosted with a second injection at day 12, consisting only of the biotinylated antigens. At day 21, the sera and spleen of mice in both groups were collected for evaluation of the humoral and cellular immune responses, respectively.

Groups		Day 0	Day 12	Day 21	Day 24
1	Control	PBS	PBS	Serum (Humoral) Spleen (Cellular)	IFN-γ Assay
2	B-GM_3 **64** B-GM_2 **65** Bfp Anti CD-40 mAb	1 μg 1 μg 20 μg 25 μg In saline	1 μg 1 μg 0 μg 0 μg In saline	Serum (Humoral) Spleen (Cellular)	IFN-γ Assay

Table 5.1 *Immunization protocol for evaluating the humoral and cell-mediated immune response to biotinylated ganglioside antigens* **64** *and* **65** *in mice. (n=5 for each group).*

The humoral immune response against the biotinylated ganglioside antigens **64** and **65** was evaluated by ELISA (Figure 5.4). Diluted serum antibody (1:1000 dilution) was incubated in microtiter plates coated with the ganglioside antigens conjugated to bovine serum albumin. Following multiple washings, the level of bound antibody was detected using a horseradish peroxidase labeled goat anti-mouse secondary antibody. The ELISA measurements were done in quadruplicate for each mouse. The mean absorbance values at 1:1,000 serum dilutions obtained for each individual mouse were further averaged in each group to generate the curve. The error bars represent the standard deviation in a group of 5 mice.

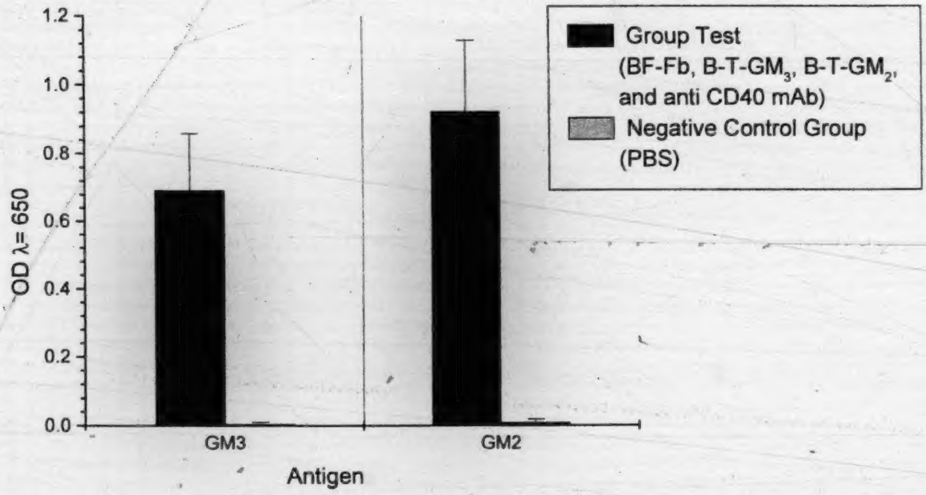


Figure 5.4 *Humoral immune response against the biotinylated gangliosides* **64** *and* **65**. *ELISA plates were coated with* GM_3*- and* GM_2*-BSA* **40** and **41** *conjugates respectively. The optical density readings are for 1:1,000 serum dilution and are average responses of five mice.*

As shown in Figure 5.4, a marked increase in serum activity was observed for mice immunized with the immunogen preparation compared to the negative control group. Further investigation of the immune response showed that a titer of ganglioside-specific antibodies greater than 20,000 was obtained in mice immunized using this dendritic-cell targeting strategy (Figure 5.5).

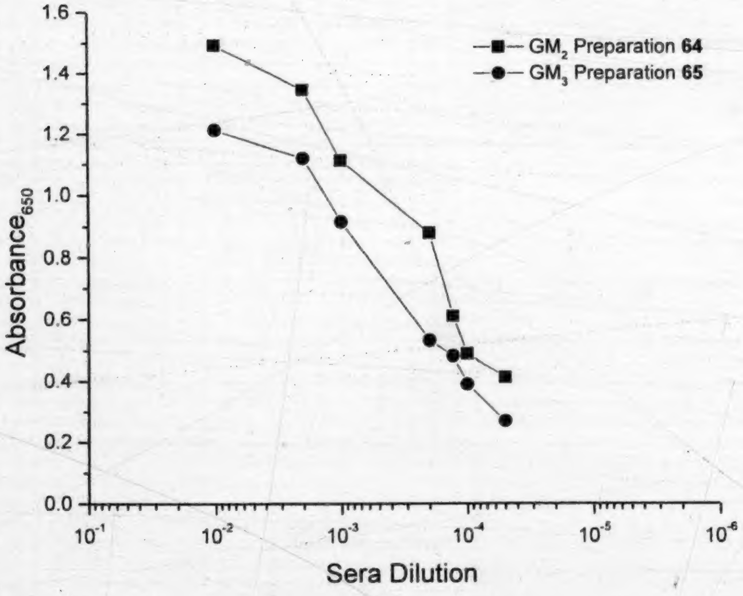


Figure 5.5 *Titration of mouse antisera derived from immunization with biotinylated GM$_3$- and GM$_2$ derivatives* **64** *and* **65**. *ELISA plates were coated with GM$_3$- and GM$_2$- BSA conjugates* **40** and **41** *respectively. Data are average responses of five mice.*

This immunization strategy whereby an immunogen is targeted to dendritic cells in the presence of an activator appeared to be a powerful approach to generate antibodies against oligosaccharide antigens. The antibody titers obtained with this approach compare favourably to the ones obtained by a standard CFA immunization protocol (Chapter 4) while avoiding conjugation to a carrier protein and injecting a significantly lower dose of antigen (50 times less). Further investigations are required to explore the type of immunoglobulin generated in the animals as well as the mode of response.

In a subsequent experiment, the cell-mediated immune response was analyzed in an indirect assay which measured IFN-γ production. Spleen cells from the immunized and negative control groups were isolated. After incubation in the presence of a stimlator cell and the corresponding antigen, the concentration of the IFN-γ in the supernatant was determined using a IFN-γ ELISA kit (eBioscience). As expected for T-cell independent antigens, the IFN-γ response was low for mice immunized with the GM_3 and GM_2 vaccine preparation (Figure 5.6)

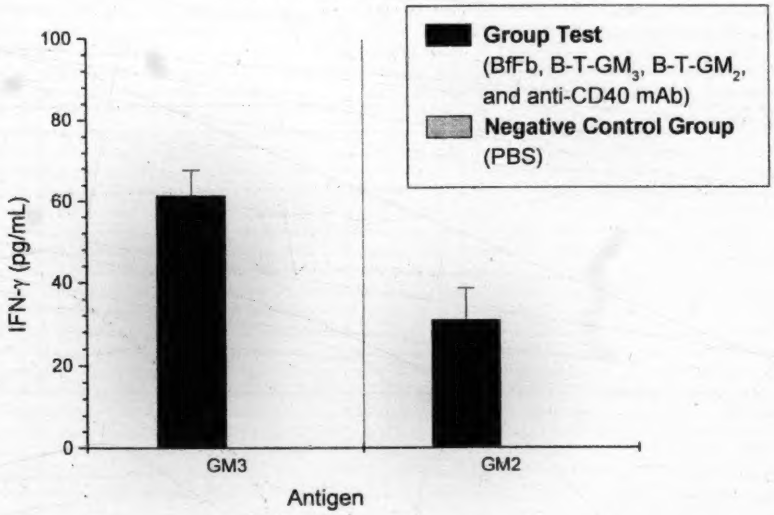


Figure 5.6 *Cellular immune response from mice immunized with biotinylated glycolipids* **64** *and* **65**. *Data are average responses of five mice.*

5.4.4 Preparation of a biotinylated glycopeptide[10]

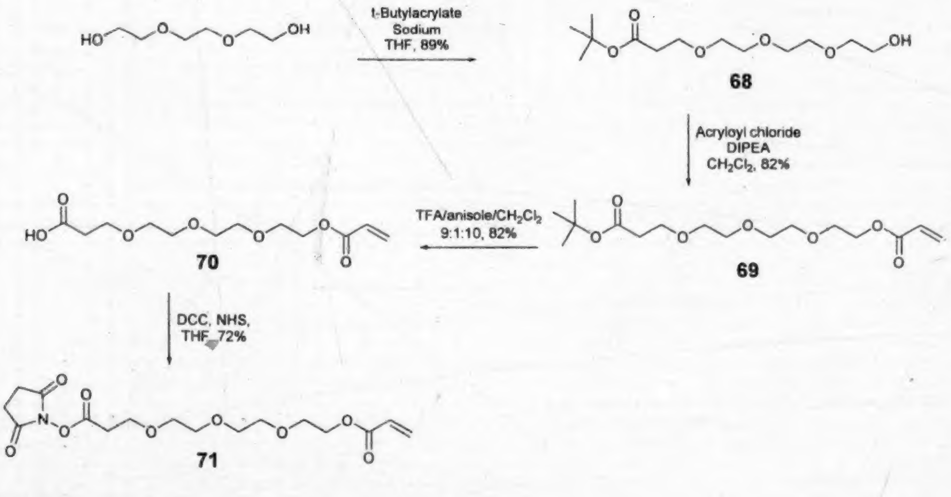
It is well know that a sustained humoral response requires T-cell involvement. As oligosaccharides are generally T-cell independent antigens that do not trigger a T-cell response, immunization with oligosaccharides usually results in low titers of low affinity antibodies. In order to solve this problem and to generate a more sustained immune response, oligosaccharides are conjugated to a carrier protein providing the T-cell peptide epitopes necessary for T-cell activation. Conjugation reactions are typically difficult to control resulting in constructs of ambiguous structure and batch to batch variations. Furthermore, a strong immune response against the carrier protein may result in suppression of oligosaccharide-specific antibodies.[274-278] To address these issues, a glycopeptide of defined structure which incorporated a B-cell epitope, the tetrasaccharide portion of ganglioside GM_2 along with a a T-cell peptide epitope and a biotin moiety was prepared and tested in our dendritic cell targeting strategy.

---

[10] The author would like to acknowledge Dr. Sebastian Dziadek for his collaboration on this synthesis.

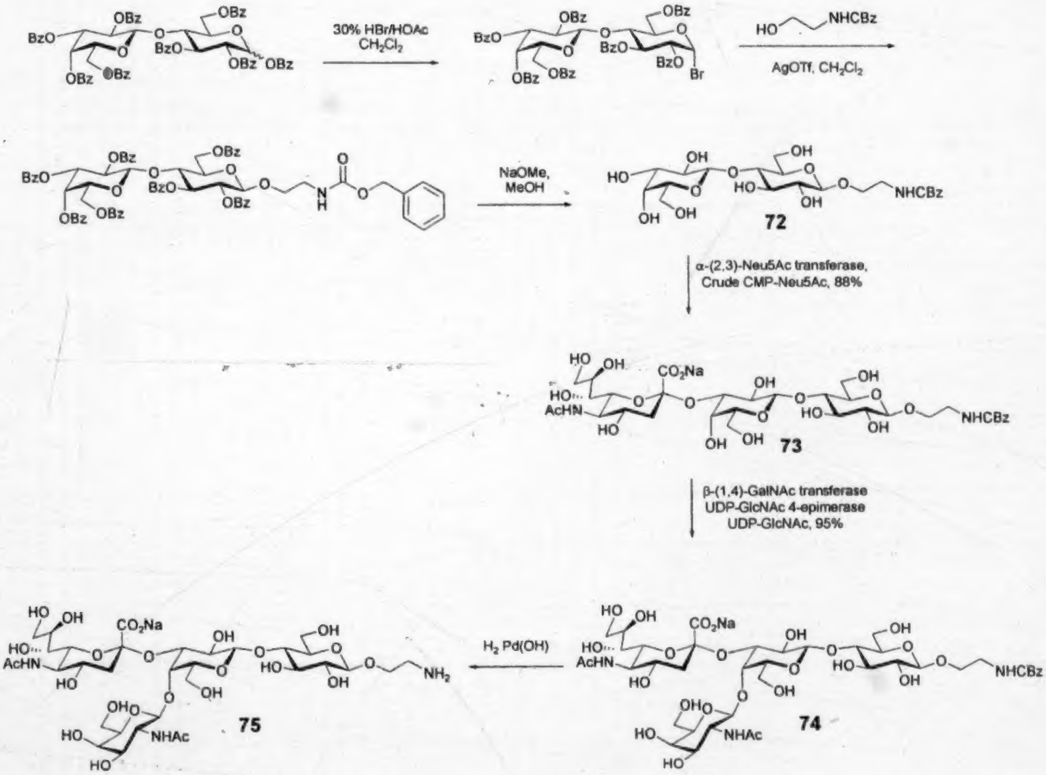
Assembly of the glycopeptide started with the development of an efficient conjugation strategy which would minimize the loss of precious oligosaccharide and peptide derivatives. A wide variety of conjugation methods have been employed to construct glycoprotein and glycopeptide conjugates.[234,279] Most of these strategies rely on the formation of reactive intermediates which are unstable to purification and often result in low reaction yield. Furthermore, several conjugation reactions result in the formation of aromatic or other cyclic structures which are potentially highly immunogenic and suppress the response against the targeted antigenic determinant.[242-244] To address these problems and minimize lost of precious building blocks, we have developed a new linker methodology for the preparation of glycoconjugates. The coupling reagent **71** is based on a water soluble non-immunogenic triethylene glycol core functionalized at one end with an amine-reactive *N*-hydroxysuccinimide activated carboxylate and incorporating at the other terminus a sulfhydryl reactive acrylate moiety.

Coupling reagent **71** was synthesized as shown in Scheme 5.3. A protected ester moiety was first inserted in triethylene glycol *via* a hetero-Michael addition of *tert*-butyl acrylate. The acrylate moiety was then appended to the molecule by reaction of **68** with acryoyl chloride under basic conditions. Deprotection of the ester followed by coupling with *N*-hydroxysuccinimide in the presence of dicyclocarbodiimide yielded the coupling reagent **71** in 72% yield.

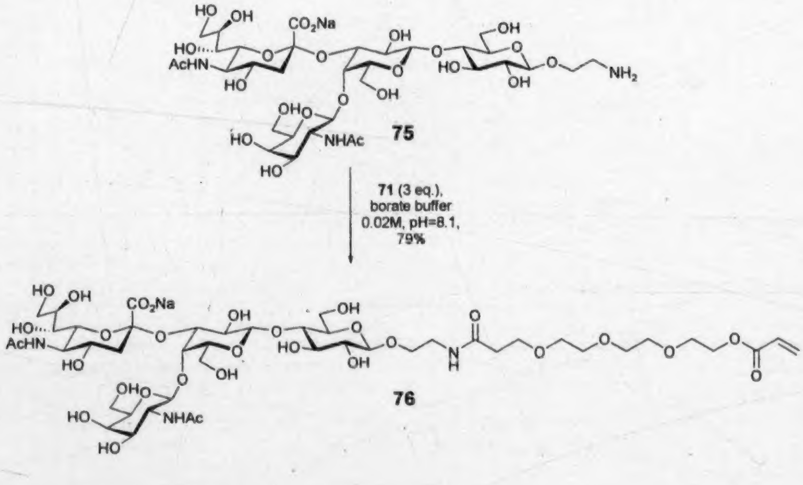


Scheme 5.3 *Synthesis of coupling reagent* **71**.

The tetrasaccharide portion of GM_2 was equipped with an amino-functionalized tether amenable for conjugation using the newly developed linker (Scheme 5.4). Lactoside **72** was synthesized according to a literature procedure from lactose and a protected aminoethanol. Perbenzoylated lactose was first treated with 30% hydrobromic acid in acetic acid to afford the lactoside bromide intermediate which was immediately glycosidated with compound a protected amino alcohol using silver triflate as a promoter to afford the corresponding lactoside. Debenzoylation of the sugar by sodium methoxide yielded intermediate **72** which was amenable to enzymatic glycosylation. Treatment of **72** with an α-(2,3)-sialyltransferase and a CMP-sialic acid preparation afforded GM_3 derivative **73** in 86% yield. After purification, the compound was incubated with a β-(1,4)-*N*-acetylgalactosaminyl transferase, a β-(1,4)-*N*-acetylglucosamine epimerase and UDP-*N*-acetylglucosamine to yield GM_2 derivative **74** in 78% yield. The GM_2 derivative was subsequently deprotected by hydrogenation to yield compound **75** with an amine handle. Incubation of the oligosaccharide derivative with coupling reagent **71** afforded a GM_2 analogue bearing a acrylate moiety for conjugation with a sulfhydryl-terminated biomolecule (Scheme 5.5).

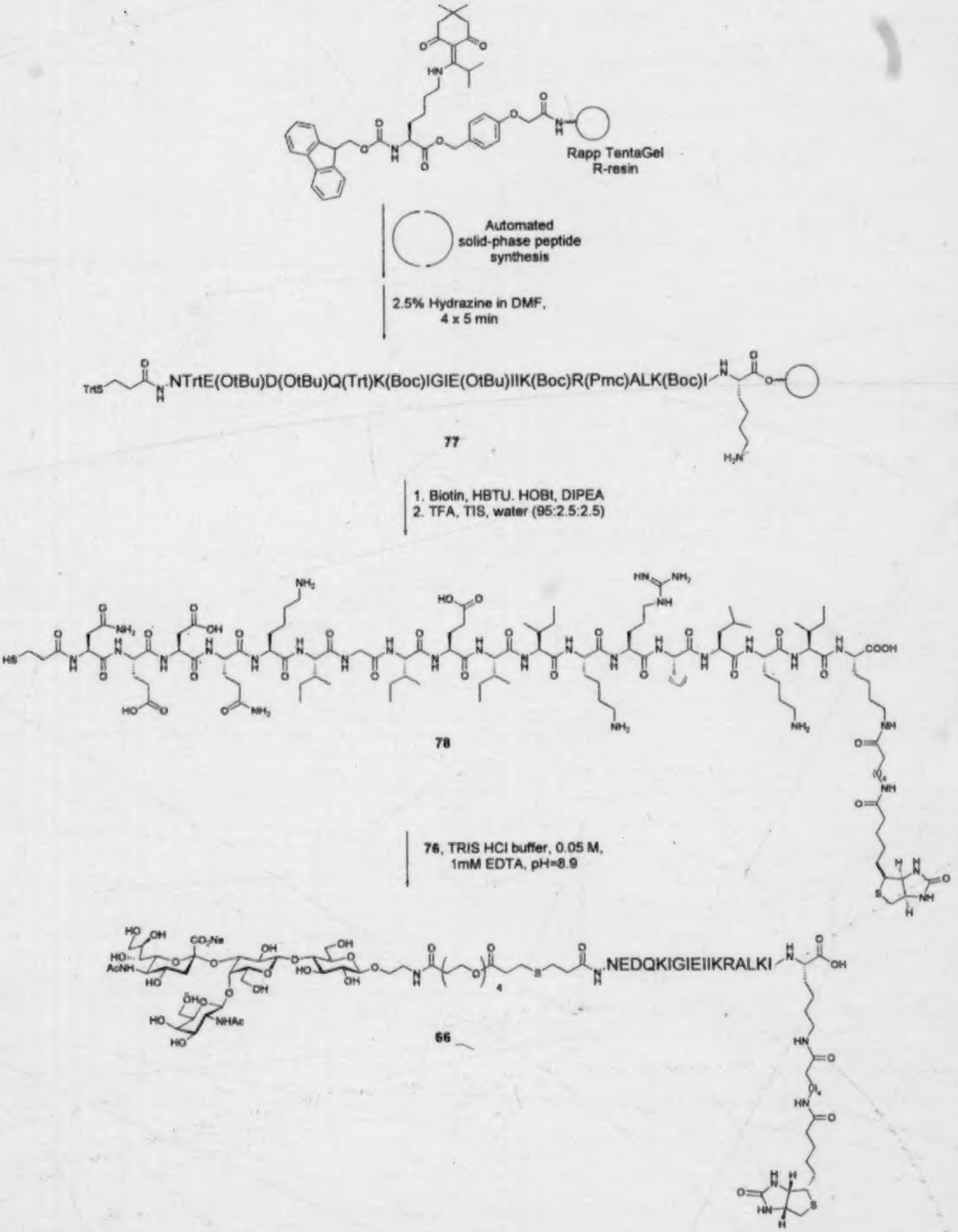

30% HBr/HOAc
CH$_2$Cl$_2$
HO NHCBz
AgOTf, CH$_2$Cl$_2$
NaOMe,
MeOH
72
α-(2,3)-Neu5Ac transferase,
Crude CMP-Neu5Ac, 88%
73
β-(1,4)-GalNAc transferase
UDP-GlcNAc 4-epimerase
UDP-GlcNAc, 95%
H$_2$ Pd(OH)
74
75

Scheme 5.4 *Synthesis of amino-fuctionalized GM$_2$ antigenic determinant.*


75
71 (3 eq.),
borate buffer
0.02M, pH=8.1,
79%
76

Scheme 5.5 *Synthesis of GM$_2$-acrylate derivative* **76**.

A peptide fragment from the murine self 60 kDa heat shock protein (hsp60) was elected as a T-cell peptide epitope.[280] This heptadecapeptide which consists of residues 458 to 472 was reported to be a potent carrier in conjugate vaccines against meningitides[281] and pneumoniae.[282] The T-cell peptide epitope was assembled using an automated peptide synthesizer employing Fmoc-protected building blocks[283] and HBTU/HOBt chemistry (Scheme 5.6).[284] The synthesis was initiated at the C-terminus with an ivDde-protected Fmoc-lysine-Fmoc-isoleucine[285,286] attached to Rapp Tentagel resin *via* an acid labile linker.[287] The orthogonally protected lysine residue provided a handle for the addition of the biotin moiety on the solid support. After completion of the peptide sequence, *S*-trityl protected mercaptopropionic acid was coupled to install a sulfhydryl moiety at the N-terminus of the peptide to serve as an attachment point for the linker. The ivDde-group was subsequently cleaved with a 35% solution of hydrazine in DMF and the free amino group coupled to biotin. The derivatized peptide was finally deprotected and liberated from the resin by treatment with an aqueous solution of trifluoroacetic acid and triisopropylsilane to obtain the target compound. The biotinylated glycopeptide was finally assembled by coupling the oligosaccharide modified with an acrylate function to the biotinylated peptide bearing a sulfhydryl group at its N-terminus. GM_2-acrylate **76** was incubated with the sulfhydryl-terminated peptide in Tris buffer, pH 8.9, containing 1 mM EDTA to prevent the formation of disulfide by-products. The biotinylated glycopeptide was obtained in 70% yield after reverse-phase HPLC purification.


Rapp TentaGel R-resin
Automated solid-phase peptide synthesis
2.5% Hydrazine in DMF, 4 x 5 min
TrtS
NTrtE(OtBu)D(OtBu)Q(Trt)K(Boc)IGIE(OtBu)IIK(Boc)R(Pmc)ALK(Boc)I
77
1. Biotin, HBTU. HOBt, DIPEA
2. TFA, TIS, water (95:2.5:2.5)
78
76, TRIS HCl buffer, 0.05 M, 1mM EDTA, pH=8.9
NEDQKIGIEIIKRALKI
66

Scheme 5.6 *Synthesis of biotinylated glycopeptide* **66**.

5.4.5 In vivo evaluation of the immune response generated by the concomitant injection of a biotinylated glycopeptide and the P125 bispecific monoclonal antibody

The immune response to a vaccine preparation comprising glycopeptide **66**, the bifunctional fusion protein and the costimulatory anti-CD40 antibody was evaluated in mice. The immunization protocol is described in detail in Table 5.2. Group 1 constituted the negative control group. The mice of this group were only injected with PBS at the different times of the immunization schedule. The second group, Group 2, was the test group. The mice of this group were immunized at day 0 with a combination of biotinylated glycopeptide **66** (1 μg), the bifunctional fusion protein (20 μg) and a co-stimulatory component, and the anti-CD40 monoclonal antibody (25 μg) in phosphate saline solution. The immune response was boosted with a second injection at day 12, consisting only of the biotinylated antigen. At day 21, the sera and spleen of mice in both groups were collected for evaluation of the humoral and cellular immune responses, respectively.

Group		Day 0	Day 12	Day 21
1	Control	PBS	PBS	Serum (Humoral)
2	B-GM_2-peptide **66** Bfp Anti CD-40 mAb	1 μg 20 μg 25 μg In saline	1 μg 0 μg 0 μg In saline	Serum (Humoral)

Table 5.2 *Immunization protocol for evaluating the humoral and cell-mediated immune response to biotinylated glycopeptide antigen* **66** *in mice. (n=5 for each group).*

The humoral immune response against the biotinylated ganglioside glycopeptide **66** was evaluated by ELISA as before. Diluted serum antibody (1:1,000 dilution) was incubated in microtiter plates coated with the ganglioside hapten conjugated to bovine serum albumin. Following multiple washings, the level of bound antibody was detected using a horseradish peroxidase labeled goat anti-mouse secondary antibody. The ELISA measurements were done in quadruplicate for each mouse and the mean absorbance values were averaged in each group of mice. Unfortunately, to date, the results of the ELISA assay were inconsistent due to problems relating to plate coating making the experimental results inconclusive.[11]

5.5 Conclusions

Biotinylated glycolipids and glycopeptides were efficiently synthesized to investigate a novel vaccination strategy in which biotinylated antigens are targeted to the endocytic pathway of dendritic cells *via* a bispecific monoclonal antibody. Mice immunized with the glycolipid preparation show a good ganglioside-specific antibody response with a minimal amount of antigen. As expected, a very low cellular immune response was detected by the INF-γ assay. Unfortunately, at the time of writing this document, inconclusive results were obtained for the biotinylated glycopeptide vaccine. Despite these results, the targeting of an antigen to dendritic cells using a bifunctional fusion protein appears to be a very promising approach to generate a strong humoral immune response against oligosaccharides.

---

[11] Personal communication with Welson Wang, Department of Pharmacy, University of Alberta.

Chapter 6
Conclusions and Future Directions

The investigations presented in this thesis led to the development of a versatile approach for the preparation of ganglioside analogues in a form suitable for conjugation. The chemoenzymatic approach was centered on a versatile lactosyl ceramide analogue which can be elaborated to gangliosides of higher complexity or functionalized with different acyl chains at the sphingosine amine. The truncated ceramide aglycon allows for coupling of the molecule *via* a variety of conjugation strategies while maintaining the relevant stereochemical and structural features of natural ceramide. Furthermore, the truncated ceramide moiety confers increased aqueous solubility allowing for enzymatic elaboration of the lactoside to complex gangliosides and simplifying manipulation of the intermediates involved.

The chemoenzymatic preparation of ganglioside analogues started with the chemical ligation of the truncated ceramide aglycon with a perbenzoylated lactosyl bromide promoted by silver trifluoromethanesulfonate. After deprotection and manipulations at the sphingosine amine, lactosyl ceramide analogue **10** was elaborated to GM_3 and GM_2 gangliosides using a combination of recombinant α-2,3-sialyltransferase and β-1,4-*N*-acetylgalactosaminyltransferase. These analogues were further derivatized by addition of differently functionalized fatty acid chains.

The glycolipids prepared by this method provided valuable intermediates for conjugate vaccine construction and evaluation. These ganglioside derivatives were conjugated to tetanus toxoid *via* a non-immunogenic adipate linker and were evaluated as antigens *in vivo*. Mice immunized with these GM_3- and GM_2-TT conjugates responded with high titers of antibodies specific for the hapten. The preponderance of the IgG isotype is indicative of an antibody class switch and T_H cell activation. Both these events suggest that the desired immunological memory was induced in the experimental animals upon vaccination. Immunochemical data obtained with these synthetic antigens revealed that elements of the ceramide moiety are recognized by antibodies induced by these conjugates.

In order to probe the influence of the ceramide moiety on the immunogenicity of the antigen, three GM_3-TT conjugates incorporating different lipids chains were prepared. It was found that the conjugates in which the protein was attached at the end of longer lipid chain induced antibody titers at least 10-fold higher than those attached closer to the glycan chain. These results suggest that glycans further away from the carrier protein are more accessible for recognition by the immune system.

In order for tumor immunotherapy to find application in the clinic, the immunogenicity of gangliosides needs to be further enhanced. In collaboration with the group of Dr. Suresh, we have developed a system whereby biotinylated ganglioside antigens are targeted to dendritic cells for improving their presentation to the immune system. Administration of the biotinylated ganglioside along with a bifunctional fusion protein and a co-stimulatory agent showed a good ganglioside-specific antibody response while using an antigen concentration 50 times less than that used in standard immunization protocols. In an effort to trigger a cellular response, a glycopeptide antigen consisting of the glycolipid linked to a known immunogenic peptide was prepared. Unfortunately, up to now, evaluation of the construct in mice yielded inconclusive results.

In the search for an effective cancer vaccine, large quantities of tumor antigens in a conjugable form are required. The chemoenzymatic preparation of gangliosides developed as part of this work is very efficient for the synthesis of small-scale of ganglioside analogues but is not as effective in providing the large quantities of material needed for a complete immunological study. We showed that a truncated lactosyl azido sphingosine can be taken up and processed by metabolically engineered bacteria to produce gangliosides. Using this technology, we prepared several complex ganglioside analogues including GM_3, GD_3, GM_2, GD_2, and GT_{1a} on a 100-500 mg scale.

It still remains an open question as to whether the antibodies generated in this study bind to gangliosides at the surface of cancer cells and how the lipid portion of the immunizing antigen influence this event. Establishment of a solid flow cytometry assay should provide answers to these questions and guide the design of subsequent vaccines. If the results of this cell-based assay are conclusive, tumor challenge experiments would ascertain that the antibodies generated by this approach are present in sufficient titers to effect clearance of tumor cells.

Although the main goal of this work was directed towards ganglioside analogues in the context of tumor immunotherapy, these molecules are not limited to vaccine applications. Through the introduction of different terminal functionalities, these ganglioside mimics may be tailored for applications that present glycolipids in ordered arrays and model membranes to study molecular recognition events. These molecules can also serve as ligands for affinity-based removal or deletion of autoantibodies in established diseases, such as Guillain-Barre syndrome.

Chapter 7
Experimental Procedures

General methods

All chemical reagents were of reagent grade and used as supplied from Sigma-Aldrich, without further purification unless otherwise indicated. Organic solvents used in reactions were obtained from a solvent purification system (Innovative Technologies Inc.) except methanol which was distilled under an inert atmosphere. Analytical thin layer chromatography (TLC) was conducted on silica gel 60-F_{254} (Merck). Plates were visualized under UV light, and/or by treatment with either acidic cerium ammonium molybdate, orcinol, ninhydrin, or 5% ethanolic sulphuric acid, followed by heating. HPLC purification was conducted using a UV absorbance detector. Separations were performed on Beckman C_{18}-silica, Kromasil C_{18}-silica or Hamilton PRP-1 reverse-phase semi-preparative columns with combinations of water and methanol as eluent (flow rate 1.5-2.0 mL min^{-1}). Size exclusion chromatography purification was performed using either Biorad P-4 Biogel resin and 5% methanol/water as eluent (flow rate 0.4 mL min^{-1}) or HW40 resin and at 60 °C and 0.1 M bicarbonate buffer, pH 8.2 as eluent.

^{1}H NMR spectra were recorded at 400, 500 or 600 MHz and ^{13}C NMR spectra were recorded at 125 or 100 MHz. Chemical shifts are reported in δ (ppm) units using ^{13}C and residual ^{1}H signals from deuterated solvents as references (δ 7.24 for $CDCl_3$, 4.80 for HOD, and 3.49 for CD_3OD). ^{1}H and ^{13}C NMR spectra were assigned with the assistance of gCOSY, HMQC, HMBC, and gTOCSY spectra. NMR data for compounds containing sphingosine/ceramide and derivatives as the aglycon were labelled as follows. Hydrogen atoms of sphingosine are denoted H-X_{sphing}, where numbering begins with H-1 at the glycosyl linkage. The *cis* and *trans* olefinic hydrogen atoms of the truncated lipid are denoted H-Xc_{sphing} and H-Xt_{sphing} respectively. Similarly, the axial and equatorial protons at the 3-position of neuraminic acid unit are denoted H-3a and H-3e respectively and the signals resulting from the hydroxyl protons of the sphingosine unit are labelled OH-1 and OH-3. For biotinylated compounds, protons of the biotin moiety were denoted H-X_{Bio}, where numbering started at the aliphatic carbonyl group. For some compounds, the coupling pattern of the H-2 of glucopyranosyl unit is reported as a multiplet, due to high order coupling.

Electrospray ionization and MALDI mass spectra were recorded on a Micromass Zabspec TOF and Voyager Elite mass spectrometer respectively by the mass spectrometry laboratory service at the University of Alberta. For high resolution mass determination, spectra were obtained by voltage scan over a narrow range at a resolution of approximately 10,000. Optical rotations were determined in a 10 cm cell at 22 ± 2°C. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}.

Enzymatic synthesis

1. CMP-Neu5Ac synthetase from *Neisseria meningitides* (NSY-05)[224]

The CMP-Neu5Ac synthetase (460Y) from *N. meningitides* was cloned in pCWori$^+$ vector downstream of a IPTG-inducible promoter. The enzyme was expressed in *Escherichia coli* AD202. The bacterial strain was grown from a glycerol stock on a YT2X agar plate containing ampicillin (150 μg/mL). After overnight incubation at 37 °C, the starter culture was initiated by inoculating a single bacterial colony in YT2X media (100 mL) containing ampicillin (150 μg/mL). The following day, a portion of the starter culture (20-40 mL depending of the optical density of the culture) was transferred to 1 L of YT2X media. The culture was incubated at 37 °C with shaking until the optical density reached 0.3. At this point, expression of the enzyme was induced by addition of IPTG (1.0 mM). The culture was then incubated at 25 °C with shaking for 24 hr from the time of induction. After this time, the cells were harvested by centrifugation at 8,000 rpm, 4°C for 20 min. The cells were resuspended in 20 mM Tris, pH 8.0 and lysed using a French press. The enzyme was isolated by centrifugation of the supernatant at 6,000 rpm, 4°C for 30 min followed by ultracentrifugation of the supernatant at 40,000 rpm, 4°C for 60 min. The supernatant, containing CMP-Neu5Ac synthetase was used to produce the CMP-Neu5Ac needed for enzymatic sialylation reactions.

2. α-(2,3)-Sialyltransferase from *Campylobacter jejuni* OH43-84 (CST-04)[188]

The α-(2,3)-Sialyltransferase from *Campylobacter jejuni* OH43-84 (Cst II) was cloned in pCWori$^+$ vector downstream of a IPTG-inducible promoter. The enzyme was expressed in *Escherichia coli* AD202. The bacterial strain was grown from a glycerol stock on a YT2X agar plate containing ampicillin (150 µg/mL). After overnight incubation at 37 °C, the starter culture was initiated by inoculating a single bacterial colony in YT2X media (100 mL) containing ampicillin (150 µg/mL). The following day, a portion of the starter culture (20-40 mL depending of the optical density of the culture) was transferred to 1 L of YT2X media. The culture was incubated at 37 °C with shaking until the optical density reached 0.3. At this point, expression of the enzyme was induced by addition of IPTG (1.0 mM). The culture was then incubated at 25 °C with shaking for 24 hr from the time of induction. After this time, the cells were harvested by centrifugation at 8,000 rpm, 4°C for 20 min. The cells were resuspended in 50 mM HEPES, pH 7.5 containing 10% (v/v) glycerol. The enzyme was isolated by centrifugation at 13,000 rpm at 4°C for 30 min followed by ultracentrifugation of the supernatant at 40,000 rpm at 4°C for 60 min. The enzyme was recovered in the pellet, which was resuspended in 50 mM HEPES, pH 7.5 containing 10% (v/v) glycerol. The suspension was used without any further purification in subsequent sialylation reactions.

3. β-(1,4)-*N*-Acetylgalactosaminyl transferase from *Campylobacter jejuni* O:36 (CJL-30)[167]

The β-(1,4)-*N*-Acetylgalactosaminyl transferase from *Campylobacter jejuni* O:36 (CgtA) was cloned in pCWori$^+$ vector downstream of a IPTG-inducible promoter. The enzyme was expressed in *Escherichia coli* AD202. The bacterial strain was grown from a glycerol stock on a YT2X agar plate containing ampicillin (150 µg/mL). After overnight incubation at 37 °C, the starter culture was initiated by inoculating a single bacterial colony in YT2X media (100 mL) containing ampicillin (150 µg/mL). The following day, a portion of the starter culture (20-40 mL depending of the optical density of the culture) was transferred to 1 L of YT2X media. The culture was incubated at 37 °C with shaking until the optical density reached 0.3. At this point, expression of the enzyme was induced by addition of IPTG (1.0 mM). The culture was then incubated at 25 °C with shaking for 24 hr from the time of induction. After this time, the cells were harvested by centrifugation at 8,000 rpm, 4 °C for 20 min. The cells were resuspended in 50 mM HEPES, pH 7.5 containing 10% (v/v) glycerol and disrupted using a French press. The enzyme was isolated by centrifugation of the supernatant at 6,000 rpm, 4 °C for 30 min followed by ultracentrifugation of the supernatant at 40,000 rpm, 4 °C for 60 min. The supernatant, containing the enzyme was used in subsequent reactions without any further purification.

4. Bifunctional UDP-GlcNAc/Glc 4-epimerase from *Campylobacter jejuni* NCTC 11168 (CPG-13)[189]

The bifunctional UDP-GlcNAc/Glc 4-epimerase from *Campylobacter jejuni* NCTC 11168 (gne) was cloned in pCWori$^+$ vector downstream of a IPTG-inducible promoter. The enzyme was expressed in *Escherichia coli* AD202. The bacterial strain was grown from a glycerol stock on a YT2X agar plate containing ampicillin (150 μg/mL). After overnight incubation at 37 °C, the starter culture was initiated by inoculating a single bacterial colony in YT2X media (100 mL) containing ampicillin (150 μg/mL). The following day, a portion of the starter culture (20-40 mL depending of the optical density of the culture) was transferred to 1 L of YT2X media. The culture was incubated at 37 °C with shaking until the optical density reached 0.3. At this point, expression of the enzyme was induced by addition of IPTG (1.0 mM). The culture was then incubated at 20 °C with shaking for 24 hr from the time of induction. After this time, the cells were harvested by centrifugation at 8,000 rpm, 4°C for 20 min. The cells were resuspended in a solution of 20 mM HEPES, pH 7.0, 200 mM NaCl, 5 mM β-mercaptoethanol and 1 mM EDTA. Pop culture reagent (Novagen) (200 μL) followed by lysonase (Novagen) (2 μL) were added. The content was vortexed and subsequently incubated for 15 min at room temperature. The mixture was centrifuged at 14,000 rpm, 4°C for 20 min. The supernatant, containing the enzyme, was collected and used in subsequent reactions without any further purification.

5. Radiochemical Assays[288]

The activity of the α-(2,3)-sialyltransferase and the β-(1,4)-*N*-acetylgalactosaminyl transferase was determined by radiochemical assays using 8-(methoxycarbonyl)octyl *O*-

(β-D-galactopyranosyl)-(1→4)-*O*-β-D-glucopyranoside and 8-(methoxycarbonyl)octyl *O*-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-β-D-glucopyranoside respectively as substrates.

The activity of the bifunctional UDP-GlcNAc/Glc 4-epimerase was determined by a radiochemical assay in which the formation of labeled UDP-GalNAc is monitored through a glycosyltransferase reaction using octyl *O*-(α-D-fucopyranosyl)-(1→2)-*O*-(β-D-galactopyranoside) as an acceptor.

One unit of enzyme activity was defined as the amount that catalyzes the formation of 1 μmol of product per minute at 37 °C using the corresponding acceptor.

6. Synthesis of CMP-Neu5Ac[224]

N-Acetyl neuraminic acid (25.0 mg; 75.5 μmol) and cytidine triphosphate, disodium salt (4.77 mg; 90.6 μmol; 1.2 eq) were suspended in 0.5 M $MgCl_2$ (2.0 mL, 50 mM in reaction) and 0.04 M dithiothreitol (100 μL; 0.2 mM in reaction). The pH was adjusted to 8.0 with 1 M NaOH solution and CMP-Neu5Ac synthetase enzyme preparation (17.9 mL) was added. The mixture was gently tumbled overnight at room temperature. The reaction mixture was then centrifuged at 14,000 rpm for 20 min, the supernatant collected and lyophilized. The crude solid obtained was used in sialylation reactions without any further purification assuming 90% conversion of the starting material.

Purification of tetanus toxoid monomer[12]

Tetanus toxoid (Staten Serum Institute; 86.5 mL; 0.8842 mg/mL) was first concentrated using an Amicon Ultra 4 spinning device (Millipore). The concentrated protein solution was then applied onto a Sephadex G-25 size exclusion column and eluted with 20 mM PBS, pH 7.2. The fractions containing the monomeric protein were then collected and concentrated using again an Amicon Ultra 4 spinning device. The concentration of the protein was determined by the BCA assay (Pierce). It was estimated that the total amount of tetanus toxoid monomer obtained after purification was 55.19 mg.

Immunization protocol 1

---

[12] The author would like to thank Adam Szpacenko for preparing the purified tetanus toxoid monomer.

BALB/c mice were immunized by interperitoneal injection of tetanus toxoid conjugates **34** and **35** (50 μg in 100 μL PBS and 100 μL Freund's complete adjuvant, homogenized). Subsequent injections (50 μg in 100 μL PBS and 100 μL Freund's incomplete adjuvant, homogenized) were made subcutaneously on days 21 and 28. Mice were bled on day 38 and the sera were isolated by centrifugation and stored at –20 °C.

Enzyme-linked immunosorbent assay (ELISA)

BSA-carbohydrate-protein conjugates (10 μg/mL in PBS) were used to coat 96-well microtiter plates (MaxiSorp, Nunc) overnight at 4 °C. The plates were washed 5 times with PBST (PBS containing 0.05% (v/v) Tween 20). Sera were diluted with PBST containing 0.1% BSA. The solutions were distributed on the coated microtiter plate and incubated at room temperature for 2 hours. The plate was washed with PBST (5 times) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1:2000 dilution in 0.1% BSA/PBST; 100 μL/well) was added. The mixture was then incubated for 1 hour. The plate was washed 5 times with PBST before addition of a 1:1 mixture 3,3',5,5'-tetramethylbenzidine (TMB) (0.4 g/L) and 0.02% H_2O_2 solution (Kirkegaard & Perry Laboratories; 100 μL/well). After 2 minutes, the reaction was stopped by addition of 1 M phosphoric acid (100 μL/well). Absorbance was read at 450 nm.

Oligosaccharide inhibition of enzyme-linked immunosorbent assay (ELISA)

Carbohydrate-protein conjugates (10 μg/mL in PBS) were used to coat 96-well microtiter plates (MaxiSorp, Nunc) overnight at 4 °C. The plates were washed 5 times with PBST (PBS containing 0.05% (v/v) Tween 20). The serum solution (1:2000 dilution in 0.1% BSA/PBST) was mixed with inhibitor dissolved in 0.1% BSA/PBST at concentrations ranging from 0.001 μM to 1 mM. The resulting solutions were added to the coated microtiter plate in triplicates, and incubated at room temperature for 2 hours. The plates were washed with PBST (5 times) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1:2000 dilution in 0.1% BSA/PBST; 100 μL/well) was added. The mixture was then incubated for 1 hour. The plates were washed 5 times with PBST before addition of a 1:1 mixture TMB (0.4 g/L) and 0.02% H_2O_2 solution (Kirkegaard & Perry Laboratories; 100 μL/well). After 2 minutes, the reaction was stopped by addition of 1 M phosphoric acid (100 μL/well). Absorbance was read at 450 nm and percent inhibition was calculated relative to wells containing sera without inhibitor.

Immunization protocol 2

A group of five Balb/c mice was immunized with a combination of biotinylated gangliosides (GM_3 (**68**) and GM_2 (**69**); 1 μg each), a bifunctional monoclonal antibody (ScFv CS fusion; 20 μg) and a costimulator (anti-CD40 monoclonal antibody; 25 μg). A control group was immunized with PBS only. A boost immunization consisting of biotinylated gangliosides (GM_3 and GM_2; 1 μg each) for the test group and PBS for the control group was administered on day 12 of the experiment. All injections were made subcutaneously near the inguinal lymph node. On day 21, the serum and spleen of the animals were collected for analysis of the humoral and cellular immune response.

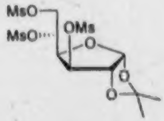
Evaluation of the humoral immune response by ELISA

Biotinylated antigens were captured by streptavidin-coated magnetic beads immobilized in microtiter plates. The plates were then washed four times with PBST and blocked with 1% BSA for 2 hr at 37 °C. After washing with PBST (four times), the diluted serum antibodies were distributed on the microtiter plate and incubated for 2 hr at room temperature. The plates were washed and bound antibodies detected by adding goat anti-mouse antibody conjugated to horseradish peroxidase (1:10 000 dilution in 1% BSA) and incubating the solution for 1 hr at room temperature. The plate was washed four times with PBST before addition of the enzyme substrate TMB. After 10 min, the absorbance was measured at 650 nm using an ELISA V_{max} kinetic microplate reader. The ELISA measurements were done in quadruplicate for each mouse. The mean ELISA values at 1:1000 serum dilutions obtained for each individual mouse were further averaged in each group to generate the graph presented in the discussion section. The error bars represent the standard deviation for the group of 5 mice.

Evaluation of the cell-mediated immune response by IFN-γ assay

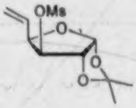
T-cells were isolated and purified from the spleen of the immunized animals and the secretion of effector molecules in the presence of stimulator cells was measured. The spleens of each group of animals were pooled and mixed in DMEM media prior to T-cell purification on a nylon wool column. Stimulator cells were isolated from naïve mice spleen cells and treated with mitomycin C (50 Ag/mL). Purified T-cells (2.5×10^5) from both test and control groups were placed in a tissue culture plate in quadruplicate with or without stimulator cells (2.5×10^6). The cells were then incubated with or without antigen (10 μg) for 3 days at 37°C under a controlled CO_2 atmosphere. After incubation, the IFN-γ concentration produced by each culture was assessed using the mouse IFN-γ ELISA Ready-SET-Go kit (eBioscience). Each data set is shown following substraction of the corresponding ELISA value obtained without stimulator cells. The error bars represent the standard deviations for each group.

Compounds

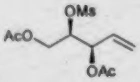


1,2-*O*-Isopropylidene-3,5,6-tri-*O*-methanesulfonyl-α-D-glucofuranose (4): 1,2-*O*-isopropylidene-α-D-glucofuranose (7.50 g; 34.1 mmol) was dissolved in anhydrous pyridine (75 mL), and the mixture was cooled to 0 °C. Methanesulfonyl chloride (10.5 mL; 136.2 mmol; 4.0 eq) was added slowly to the solution and the mixture was allowed to warm up to room temperature slowly overnight. The reaction mixture was poured to a mixture of ice/water (60 mL) with vigorous stirring. The precipitate was filtered off, and the white solid was suspended in water (30 mL) again with vigorous stirring. The precipitate was filtered off and washed with more water (30 mL), and air-dried to afford 4 (15.06 g; 33.1 mmol; 97% yield): ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 5.92 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 5.08 (d, 1H, $J_{3,2}$ = 2.6 Hz, H-3), 5.04 (ddd, 1H, $J_{5,4}$ = 8.9 Hz, $J_{5,6b}$ = 4.4 Hz, $J_{5,6a}$ = 1.3 Hz, H-5), 4.95 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-2), 4.67 (dd, 1H, $J_{6a,6b}$ = 12.1 Hz, $J_{6a,5} \approx$

1 Hz, H-6a), 4.46 (dd, 1H, $J_{4,5}$ = 8.9 Hz, $J_{4,3}$ = 2.8 Hz, H-4), 4.40 (dd, 1H, $J_{6a,6b}$ = 12.1 Hz, $J_{6b,5}$ = 4.6 Hz, H-6b), 3.17 (s, 3H, CH_3SO_2), 3.15 (s, 3H, CH_3SO_2), 3.05 (s, 3H, CH_3SO_2), 1.48 (s, 3H, isopropylidene), 1.30 (s, 3H, isopropylidene).

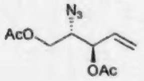


5,6-Dideoxy-1,2-*O*-isopropylidene-3-*O*-methanesulfonyl-α-D-*xylo*-hexose-5-ene (5): The trimesylate **4** (18.6 g, 40.9 mmol) was dissolved in DMF (90 mL), potassium iodide (26.2 g, 157.8 mmol; 3.85 eq) was added and the mixture was heated to 90 °C with stirring for 10 hr. TLC showed that the starting material was completely converted. The mixture was cooled down to room temperature and the solvent was removed under reduced pressure. The dark residue was suspended in ethyl acetate (600 mL) and washed with an aqueous solution of sodium thiosulfate (30 g dissolved in 150 mL). The almost colorless organic solution was separated and dried over anhydrous sodium sulphate and evaporated to dryness to give the desired alkene **5** (10.67 g; 40.37 mmol; 99%). R_f: 0.29 (EtOAc/toluene 1:9). $[\alpha]_D$ -17.7° (*c* 1.8, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ_H 5.95 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1), 5.83 (ddd, 1H, $J_{5,6t}$ = 17.3 Hz, $J_{5,6c}$ = 10.5 Hz, $J_{5,4}$ = 6.4 Hz, H-5), 5.47 (ddd, 1H, $J_{6t,5}$ = 17.4 Hz, $J_{6t,6c} \approx J_{6t,4} \approx$ 1.3 Hz, H-6_trans), 5.36 (ddd, 1H, $J_{6c,5}$ = 10.5 Hz, $J_{6c,6t} \approx J_{6c,4} \approx$ 1.2 Hz, H-6_cis), 4.94 (d, 1H, $J_{3,4}$ = 2.9 Hz, H-3), 4.77 (d, 1H, $J_{2,1}$ = 3.8 Hz, H-2), 4.75 (m, 1H, H-4), 3.00 (s, 3H, CH_3SO_2), 1.50 (s, 3H, isopropylidene), 1.31 (s, 3H, isopropylidene); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 130.0 (C-5), 120.0 (C-6), 112.0 ($C(CH_3)_2$, isopropylidene), 104.1 (C-1), 83.2 (C-2), 82.7 (C-3), 79.4 (C-4), 38.0 (CH_3SO_2), 26.2 (CH_3_isopropylidene), 25.8 (CH_3_isopropylidene). ESI HRMS calc'd for $C_{10}H_{16}O_7SNa$: 264.0668. Found: 264.0664.

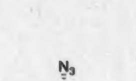


(2*R*,3*R*)-1,3-Di-*O*-acetyl-2-*O*-methanesulfonyl-penta-4-ene-1,2,3-triol (7): Compound **5** (5.41 g; 20.5 mmol) was dissolved in a mixture of trifluoroacetic acid (20 mL) and water (20 mL) and the solution was heated to 55 °C for 2 h. The mixture was concentrated under reduced pressure and co-evaporated with toluene to give the hemi-acetal as syrup. The above syrup was dissolved in water (25 mL) and the pH of the solution was adjusted to 8.5 using a saturated solution of $NaHCO_3$. The reaction mixture was placed in an ice bath, and an aqueous solution of $NaIO_4$ (6.57 g dissolved in 25 mL of water, 30.8 mmol; 1.5 eq) was added slowly into the solution and the reaction was stirred for 90 min until TLC (CH_2Cl_2/MeOH 95:5) indicated complete consumption of the hemiacetal. The solution was then extracted with EtOAc (3 × 50 mL). The organic phase was collected, dried over anhydrous anhydrous sodium sulphate, and concentrated to give a syrup. This syrup was dissolved in ethanol (100 mL) and cooled to 0° C. Sodium borohydride (1.55 g; 41.0 mmol; 2.0 eq) was added by small portions. The reaction was stirred at 0° C for 1.5 h, left at room temperature for 1 h, and quenched by slow addition of AcOH (10 mL). The reaction was concentrated under reduced pressure while keeping the bath temperature below 30 °C to give a white solid. The solid was triturated with EtOAc (3 × 25 mL) and the insoluble material was removed by filtration. The filtrate was concentrated to give colorless syrup (**6**) under reduced pressure by keeping the bath temperature below 30 °C. A pure sample of **6** can be obtained by chromatography on silica gel using MeOH-CH_2Cl_2 as eluent (0 → 3%). R_f: 0.59 (MeOH/CH_2Cl_2: 1:9): $[\alpha]_D$ +10.1° (*c* 0.7, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.86 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 6.0 Hz, H-4), 5.41 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 2.5 Hz, $J_{5t,3}$ = 1.3 Hz, H-5t), 5.29 (ddd, 1H, $J_{5c,4}$ = 10.5 Hz, $J_{5c,5t}$ = 2.5 Hz, $J_{5c,3}$ = 1.3 Hz, H-5c), 4.58 (m, 1H, H-2), 4.38 (m, 1H, H-3), 3.90 (ddd, 1H, $J_{1a,1b}$ = 12.7 Hz, $J_{1a,2}$ = 3.5 Hz, $J_{1a,3}$ = 1.3 Hz, H-1a), 3.79 (ddd, 1H, $J_{1b,1a}$ = 12.7 Hz, $J_{1b,2}$ = 6.0 Hz, $J_{1b,3}$ = 1.3 Hz, H-1b), 3.13-3.21 (m, 2H, OH-1 + OH-3), 3.11 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 135.3, 118.4, 85.1, 71.7, 61.9, 38.6. A mixture of acetic anhydride (25 mL) and pyridine (25 mL) was added to the mixture and the reaction was stirred at room temperature overnight. After concentration, ethyl acetate (1000 mL) was added, and the mixture was washed with brine (2 × 15 mL), dried over anhydrous anhydrous sodium sulfate and concentrated. The compound **7** (4.08 g; 14.6 mmol; 71%) was finally obtained by chromatography on silica gel using toluene/EtOAc (9:1) as eluent: R_f: 0.29 (EtOAc/toluene: 2:8): $[\alpha]_D$ +30.0° (*c* 1.3, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.74

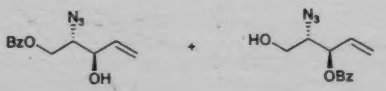
(ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 6.6 Hz, H-4), 5.43 (dddd, 1H, $J_{3,4} \approx J_{3,2} \approx$ 6.4 Hz, $J_{3,5t} \approx J_{3,5c} \approx$ 1.1 Hz, H-3), 5.38 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 1.1 Hz, H-5t), 5.32 (ddd, 1H, $J_{5c,4}$ = 10.5 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 1.1 Hz, H-5c), 4.83 (ddd, 1H, $J_{2,3} \approx J_{2,1b} \approx$ 6.7 Hz, $J_{2,1a}$ = 3.1 Hz, H-2), 4.34 (dd, 1H, $J_{1a,1b}$ = 12.4, $J_{1a,2}$ = 3.1 Hz, H-1a), 4.05 (dd, 1H, $J_{1b,1a}$ = 12.4, $J_{1b,2}$ = 7.0 Hz, H-1b), 3.01 (s, 3H, CH_3SO_2), 2.05 (s, 3H, Ac), 2.03 (s, 3H, Ac); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 170.1, 169.3, 130.7, 120.8, 78.8, 72.0, 62.2, 38.6, 20.7, 20.5. ESI HRMS calc'd for $C_{10}H_{16}O_7SNa$: 303.0514. Found: 303.0514. Anal. calc'd for $C_{10}H_{16}O_7S$: C, 42.85; H, 5.75. Found: C, 42.54; H, 5.50.



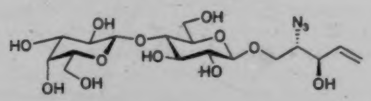
(2*S*,3*R*)-1,3-Di-*O*-acetyl-2-azido-penta-4-ene-1,3-diol (8): To a solution of mesylate **7** (4.08 g; 14.6 mmol) in anhydrous DMF (40 mL) was added NaN_3 (3.78 g; 58.2 mmol; 4.0 eq). The mixture was stirred overnight at 95 °C. The mixture was then cooled and concentrated to 5 mL. The solution was then diluted with AcOEt (50 mL), washed with brine (2 x 15 mL), dried over anhydrous sodium sulfate and concentrated. Chromatography on silica gel using EtOAc/toluene (5:95) as eluent afforded compound **8** as a colorless syrup (1.87 g; 8.16 mmol; 56%): R_f: 0.37 (EtOAc/toluene: 2:8). $[\alpha]_D$ -16° (*c* 0.6, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 5.80 (high order ddd, 1H, $J_{4,5t}$ = 17.5 Hz, $J_{4,5c}$ = 10.7 Hz, $J_{4,3}$ = 6.8 Hz, H-4), 5.30 - 5.37 (m, 3H, H-3, H-5t, H-5c), 4.12 (dd, 1H, $J_{1a,1b}$ = 11.7 Hz, $J_{1a,2}$ = 4.5 Hz, H-1a), 4.06 (dd, 1H, $J_{1b,1a}$ = 11.7 Hz, $J_{1b,2}$ = 7.7 Hz, H-1b), 3.80 (ddd, 1H, $J_{2,1b}$ = 7.8 Hz, $J_{2,1a} \approx J_{2,3} \approx$ 4.6 Hz, H-2), 2.06 (s, 3H, OAc), 2.05 (s, 3H, OAc). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 170.4, 169.4, 131.3, 120.5, 73.6, 62.8, 62.2, 20.8, 20.6. ESI HRMS calc'd for $C_9H_{13}N_3O_4Na$: 250.0804. Found: 250.0801. Anal. calc'd for $C_9H_{13}N_3O_4$: C, 47.57; H, 5.77; N, 18.49. Found: C, 47.66; H, 5.89; N, 18.50.



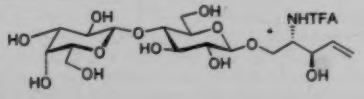
(2*S*,3*R*)-2-Azido-penta-4-ene-1,3-diol (1): A solution of sodium methoxide in methanol (1.0 M, 1 mL) was added to a solution of compound **8** (1.87 g; 8.16 mmol) in anhydrous methanol (10 mL). The mixture was stirred at room temperature for 2 h. After this time, the solution was neutralized with acidic ion-exchange resin (Amberlite IR-120), the resin was filtered off and the filtrate was concentrated under reduced pressure to give **1** (1.17 g; 8.16 mmol; quantitative) as a colorless syrup. This compound should be stored at -20 °C: R_f: 0.17 (EtOAc/toluene 3:7). $[\alpha]_D$ -7.7° (*c* 1.3, MeOH). ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 5.95 (ddd, 1H, $J_{4,5t} = 17.0$ Hz, $J_{4,5c} = 10.5$ Hz, $J_{4,3} = 6.2$ Hz, H-4), 5.39 (ddd, 1H, $J_{5t,4} = 17.2$ Hz, $J_{5t,5c} \approx J_{5t,3} \approx 1.3$ Hz, H-5t), 5.29 (ddd, 1H, $J_{5c,4} = 10.4$ Hz, $J_{5c,5t} \approx J_{5c,3} \approx 1.3$ Hz, H-5c), 4.29 (m, 1H, H-3), 3.74-3.85 (m, 2H, H-1a, H-1b), 3.49 (ddd, 1H, $J_{2,1a} \approx J_{2,1b} \approx J_{2,3} \approx 5.2$ Hz, H-2), 2.41 (br s, 2H, OH-1, OH-3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 136.4, 118.1, 73.5, 66.3, 62.3. ESI HRMS calc'd for $C_5H_9N_3O_2Na$: 166.0593. Found: 166.0592. Anal. calc'd for $C_5H_9N_3O_2$: C, 41.95; H, 6.34; N, 29.36. Found: C, 41.59; H, 6.41; N, 29.48.



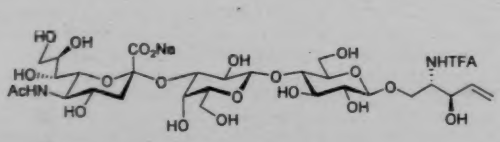
(2*S*,3*R*)-2-azido-1-*O*-benzoyl-penta-4-ene-1,3-diol (9) and (2*S*,3*R*)-2-azido-3-*O*-benzoyl-penta-4-ene-1,3-diol (10): To a solution containing diol (**1**) (1.17 g; 8.17 mmol) and trimethyl orthobenzoate (2.46 mL; 14.3 mmol; 1.75) in chloroform (20 mL), was added camphorsulphonic acid (94.9 mg; 0.409 mmol; 0.05 eq). The reaction mixture was concentrated to 1/3 of its original volume using a rotary evaporator. As TLC revealed that the reaction was not completed, more chloroform (~5 mL) was added to the mixture, and the solution was again concentrated to 1/3 of its volume. This cycle was repeated until TLC indicated complete consumption of the starting material (usually 3 cycles were needed). Once the reaction was complete, triethylamine (100 µL) was added to the reaction mixture and the solvent was removed completely. The resulting syrup was treated then with a 20% AcOH-H_2O solution (10 mL). After stirring for 30 min, the reaction mixture was concentrated to dryness. The syrupy mixture was subsequently purified by column chromatography on silica gel using EtOAc/toluene (5:95) as eluent to afford **9** (1.01 g; 4.09 mmol; 50%) and **10** (0.909 g; 3.68 mmol; 45%). Data for **9**: R_f: 0.23 (EtOAc/toluene 1:9). $[\alpha]_D$ -1.6° (*c* 0.7, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ_H 8.02 (m, 2H, Bz), 7.53 (m, 1H, Bz), 7.41 (m, 2H, Bz), 5.93 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.6 Hz, $J_{4,3}$ = 6.5 Hz, H-4), 5.40 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 1.3 Hz, H-5t), 5.28 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 1.3 Hz, H-5c), 4.56 (dd, $J_{1a,1b}$ = 12.1 Hz, $J_{1a,2}$ = 3.7 Hz, H-1a), 4.40 (dd, 1H, $J_{1b,1a}$ = 11.8 Hz, $J_{1b,2}$ = 7.9 Hz, H-1b), 4.27 (m, 1H, H-3), 3.79 (m, 1H, H-2); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 166.3, 135.7, 133.2, 129.6 (2C), 129.2, 128.3, 118.2, 72.3, 64.4, 64.1. ESI HRMS calc'd for $C_{12}H_{13}N_3O_3Na$: 270.0855. Found: 270.0846. Anal. calc'd for $C_{12}H_{13}N_3O_3$: C, 58.29; H, 5.30; N, 17.00. Found: C, 58.13; H, 5.21; N, 16.44. Data for **10**: R_f: 0.18 (EtOAc/toluene 1:9). $[\alpha]_D$ -18.6° (*c* 0.6, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ_H 8.05 (m, 2H, Bz), 7.57 (m, 1H, Bz), 7.44 (m, 2H, Bz), 5.97 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.6 Hz, $J_{4,3}$ = 6.8 Hz, H-4), 5.66 (m, 1H, H-3), 5.48 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 1.1 Hz, H-5t), 5.35 (ddd, 1H, $J_{5c,4}$ = 10.6 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 0.9 Hz, H-5c), 3.83 (ddd, 1H, $J_{2,1b}$ = 7.7 Hz, $J_{2,1a} \approx J_{2,3} \approx$ 4.7 Hz, H-2), 3.78 (m, H-1a), 3.67 (m, 1H, H-1b), 2.94 (br t, 1H, J = 5.7 Hz, OH-1); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 165.4 , 133.3, 131.5, 129.6, 129.3, 128.4 (2C), 120.3, 74.4, 65.7, 61.6. ESI HRMS calc'd for $C_{12}H_{13}N_3O_3Na$: 270.0855. Found: 270.0848. Anal. calc'd for $C_{12}H_{13}N_3O_3$: C, 58.29; H, 5.30; N, 17.00. Found: C, 57.87; H, 5.15; N, 16.73.



***O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-azido-penta-4-ene-1,3-diol (11):** Perbenzoylated lactosyl bromide (4.37 g; 3.85 mmol) and selectively protected azidosphingosine **9** (1.0 g; 4.05 mmol; 1.05) was dissolved in anhydrous toluene (25 mL). Molecular sieves (4 Å; 1.0 g) was added and the mixture was stirred for 10 min at room temperature. After cooling the reaction to –78 °C, silver triflate (1.49 g; 5.78 mmol; 1.50 eq) was added, and the reaction mixture was allowed to warm up to 20 °C within 2 h. After this time, the reaction mixture was diluted with more toluene (50 mL) and the insoluble material was filtered off. The organic phase was then washed with a 10% aqueous ammonia solution (2 x 25 mL), dried over anhydrous sodium sulphate, and evaporated to afford a crude syrup. Anhydrous MeOH (25 mL) was added the crude syrup under an argon atmosphere, and a solution of NaOMe in MeOH (~1.0 M, 2 mL) was added, the mixture was stirred at 60°C until TLC indicated complete debenzoylation. The reaction was then cooled to room temperature and the solution was neutralized with acidic resin (Amberlite IR-120). The resin was filtered off and the filtrate concentrated. Purification of the resulting crude solid by reverse phase chromatography afforded **11** as a white solid (1.51 g; 3.23 mmol; 84%): R_f: 0.17 (CH_2Cl_2/MeOH/H_2O 8:2:0.2). $[\alpha]_D$ -4.8° (*c* 0.4, MeOH). ^{1}H NMR (600 MHz, CD_3OD): δ_H 5.91 (ddd, 1H, $J_{4,5t}$ = 17.1 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 6.6 Hz, H-4$_{\text{sphing}}$), 5.34 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 1.6 Hz, H-5t$_{\text{sphing}}$), 5.22 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 1.3 Hz, H-5c$_{\text{sphing}}$), 4.35 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1$_{\text{Gal}}$), 4.31 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1$_{\text{Glc}}$), 4.22 (dd, 1H, $J_{3,4}$ = 6.4 Hz, $J_{3,2}$ = 5.3 Hz, H-3$_{\text{sphing}}$), 3.89-3.94 (m, 2H, H-6a$_{\text{Glc}}$, H-1a$_{\text{sphing}}$), 3.84 (dd, 1H, $J_{6b,6a}$ = 11.6 Hz, $J_{6b,5}$ = 4.6 Hz, H-6b$_{\text{Glc}}$), 3.81 (br d, 1H, $J_{4,3}$ = 3.3 Hz, H-4$_{\text{Gal}}$), 3.77 (dd, 1H, $J_{6a,6b}$ = 11.5 Hz, $J_{6a,5}$ = 7.5 Hz, H-6a$_{\text{Gal}}$), 3.72 (dd, 1H, $J_{1b,1a}$ = 10.4 Hz, $J_{1b,2}$ = 4.2 Hz, H-1b$_{\text{sphing}}$), 3.69 (dd, 1H, $J_{6b,6a}$ = 11.5 Hz, $J_{6b,5}$ = 4.8 Hz, H-6b$_{\text{Gal}}$), 3.67 (m, 1H, H-2$_{\text{sphing}}$), 3.59 (m, 1H, H-5$_{\text{Gal}}$), 3.56 (t, 1H, *J* = 10.1 Hz, H-4$_{\text{Glc}}$), 3.51-3.55 (m, 3H, H-2$_{\text{Gal}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$), 3.48 (dd, 1H, $J_{3,2}$ = 9.7 Hz, $J_{3,4}$ = 3.3 Hz, H-3Gal), 3.42 (ddd, 1H, $J_{4,5}$ = 9.7 Hz, $J_{5,6b}$ = 4.4 Hz, $J_{5,6a}$ = 2.6 Hz, H-5$_{\text{Glc}}$), 3.27 (dd, 1H, $J_{2,1}$ = 9.2 Hz, $J_{2,3}$ = 8.1 Hz, H-2_Glc); ^{13}C NMR (100 MHz, CD_3OD): δ_C 138.2, 118.0, 105.2, 104.4, 80.7, 77.2, 76.7, 76.5, 74.9, 74.8, 73.8, 72.7, 70.4, 70.1, 67.0, 62.6, 62.0. ESI HRMS calc'd for $C_{17}H_{29}N_3O_{12}Na$: 490.1649. Found: 490.1648.

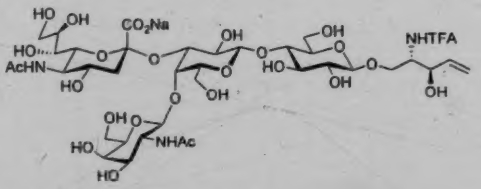


O-(β-D-Galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(trifluoroacetamido)-pent-4-ene-1,3-diol (12). The lactosyl azidosphingosine **11** (151.5 mg; 324 μmol) was dissolved in a solution of pyridine/water/triethylamine (10:1:0.3; 11.3 mL). The solution was saturated with H_2S by bubbling the gas into the solution for 1 hr at 0°C, followed by stirring for 36 hr. The solvent was evaporated and the residue was dissolved in water. After addition of acetic acid (200 μL), the mixture was applied to a Sep-Pak (C18; 5g). The salt obtained was then eluted through Amberlite IRA-400 (Cl^-) resin preconditioned with 2 M NaOH yielding the free amine (124.7 mg; 282 μL; 87%). A portion of the material (76.8 mg; 174 μmol) was dissolved in dry degassed methanol (5.0 mL) and methyl trifluoroacetate (334.0 mg; 2.61 mmol; 15 eq) was added. The reaction mixture was stirred at room temperature overnight. The solution was then concentrated. The residue was redissolved in water and applied to a Sep-Pak (C18; 5 g). Gradient elution (MeOH-H_2O) afforded **12**. A sample (110.0 mg; 249.0 μmol) was further purified by HPLC (100.0 mg; 186.1 μmol; 75%): $[\alpha]_D$ +9.1° (*c* 0.55, H_2O). ^1H NMR (500 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.34 (ddd, 1H, $J_{5t,4}$ = 17.3 Hz, $J_{5t,3}$ = 1.1 Hz, $J_{5t,5c}$ = 1.1 Hz, H-$5t_{\mathrm{sphing}}$), 5.29 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.1 Hz, $J_{5c,3}$ = 1.1 Hz, H-$5c_{\mathrm{sphing}}$), 4.48 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Glc}), 4.44 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 4.31 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 7.0 Hz, H-3_{sphing}), 4.30 (dd, 1H, $J_{1a,1b}$ = 7.0 Hz, $J_{1a,2}$ = 7.0 Hz, H-$1a_{\mathrm{sphing}}$) 4.22 (ddd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 7.0 Hz, H-2_{sphing}), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.1 Hz, H-$6a_{\mathrm{Glc}}$), 3.92-3.89 (m, 2H, H-4_{Gal}, H-$1b_{\mathrm{sphing}}$), 3.81-3.70 (m, 4H, H-$6b_{\mathrm{Glc}}$, H-5_{Gal}, H-$6a_{\mathrm{Gal}}$, H-$6b_{\mathrm{Gal}}$), 3.67-3.57 (m, 4H, H-3_{Gal}, H-3_{Glc}, H-4_{Glc}, H-5_{Glc}), 3.53 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 7.9 Hz, H-2_{Gal}), 3.32 (m, 1H, H-2_{Glc}); ^{13}C NMR (125 MHz, D_2O): δ_C 160.0 (q, $J_{C,F}$ = 37 Hz), 136.4, 119.4, 116.7 (q, $J_{C,F}$ = 286 Hz), 103.8, 103.0, 79.3, 76.2, 75.7, 75.1, 73.6, 73.4, 72.4, 71.8, 69.4, 68.6, 61.9, 61.0, 54.8. ESI HRMS calc'd for $C_{19}H_{30}F_3NO_{13}Na$: 560.1562. Found: 560.1559.



***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-trifluoroacetamido-pent-4-ene-1,3-diol (2).** The acceptor **12** (13.6 mg; 25.2 μmol; 5.04 mM in reaction), crude CMP-Neu5Ac (~45.3 μmol; ~9.06 mM in reaction; ~1.8 eq), $MnCl_2$ (100 μL of 0.5 M solution; 10 mM in reaction), $MgCl_2$ (100 μL of 0.5 M solution; 10 mM in reaction), dithiothreitol (125 μL of 0.1 M solution; 2.5 mM in reaction), water

(600 μL), α-(2,3)-sialyltransferase (4.5 mL; ~4.5 U), and alkaline phosphatase (Roche Bioscience) (2.5 μL; ~2.5 U) in 0.05 M HEPES buffer, pH 7.5 containing 10% (v/v) glycerol were combined in a 15 mL Falcon tube. The mixture was tumbled gently at room temperature for 24 hr after which time TLC ($CHCl_3$-MeOH-H_2O-AcOH; 10:7:1:2) indicated almost complete disappearance of the starting material **12**. The reaction mixture was centrifuged at 14,000 rpm for 30 min and the supernatant applied to a Sep-Pak column (C18; 10 g). Gradient elution (MeOH-H_2O) afforded **2**. Further purification by size exclusion chromatography (Biogel P-4 resin; 50 cm × 2 cm) using 5% MeOH-H_2O as an eluent yielded **2** (18.9 mg; 22.2 μmol; 88%): $[\alpha]_D$ +5.1° (*c* 1.08, H_2O). ^1H NMR (500 MHz, D_2O): δ_H 5.83 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.2 Hz, H-4$_{sphing}$), 5.33 (m, 1H, H-5t$_{sphing}$), 5.28 (m, 1H, H-5c$_{sphing}$), 4.51 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{Gal}$), 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.30 (dd, 1H, $J_{3,2}$ = 7.2 Hz, $J_{3,4}$ = 7.2 Hz, H-3$_{sphing}$), 4.22 (ddd, 1H, $J_{2,3}$ = 7.1 Hz, $J_{2,1a}$ = 7.1 Hz, $J_{2,1b}$ = 3.6 Hz, H-2$_{sphing}$), 4.11-4.07 (m, 2H, H-1a$_{sphing}$, H-3$_{Gal}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 2.1 Hz, H-6a$_{Glc}$), 3.94 (d, 1H, $J_{4,3}$ = 3.05 Hz, H-4$_{Gal}$), 3.91-3.79 (m, 9H, H-1b$_{sphing}$, H-8$_{Neu}$, H-9a$_{Neu}$, H-5$_{Gal}$, H-9b$_{Neu}$, H-6$_{Neu}$, H-7$_{Neu}$, H-2$_{Gal}$, H-6b$_{Glc}$), 3.75-3.56 (m, 7H, H-4$_{Neu}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{Neu}$, H-3$_{Glc}$, H-4$_{Glc}$, H-5$_{Glc}$), 3.32 (m, 1H, H-2$_{Glc}$), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.2 Hz, $J_{3e,4}$ = 4.7 Hz, H-3e$_{Neu}$), 2.02 (s, 3H, C(O)$\underline{CH_3}$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.2 Hz, $J_{3a,4}$ = 12.2 Hz, H-3a$_{Neu}$); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 174.7, 160.0, 136.4, 119.4, 116.7 (q, $J_{C,F}$ = 285 Hz), 103.6, 100.7, 79.2, 76.4, 76.1, 75.7, 75.1, 73.8, 73.6, 72.7, 72.4, 70.2, 69.2, 69.0, 68.6, 68.4, 63.5, 61.9, 60.9, 54.8, 52.6, 49.8, 40.5, 22.9. ESI HRMS calc'd for $C_{30}H_{46}N_2O_{21}F_3$: 827.2545. Found: 827.2551.

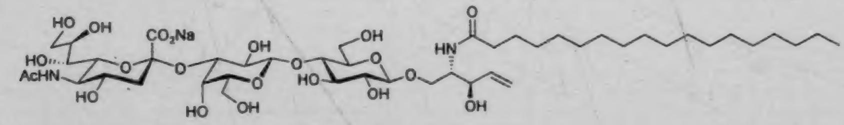


***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-**

trifluoroacetamido-pent-4-ene-1,3-diol (3). The acceptor **2** (41.6 mg, 50.2 μmol) and UDP-*N*-acetylglucosamine disodium salt (49.0 mg; 75.2 μmol; 1.5 eq) were combined in a Falcon tube. UDP-*N*-acetylglucosamine 4-epimerase (1.0 mL; ~1.0 U) in 0.2 M NaCl, 1.0 mM EDTA, 20 mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 and *N*-acetylgalactosaminyl transferase (2.94 mL; ~2.5 U) were added to the mixture followed by 48 μL of 0.5 M $MgCl_2$ (6.0 mM in reaction) and alkaline phosphatase (10 μL; ~10 U). The reaction was gently tumbled for 16 hr until the reaction was completed as observed by silica gel TLC (EtOAc-MeOH-H_2O-AcOH; 6:3:3:2). The mixture was centrifuged for 20 min at 14,000 rpm, the supernatant was collected and applied to a Sep-Pak (C18; 5 g). Elution with a gradient of water-methanol afforded **3**. A portion (18.5 mg, 17.9 μmol) was further purified by size exclusion chromatography (Biogel P-4 resin; 50 cm × 2 cm) and eluted with 5% MeOH/H_2O to yield **3** (17.7 mg, 17.2 μmol, 95%): $[\alpha]_D$ +14.2° (*c* 0.32, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{\text{sphing}}$), 5.33 (m, 1H, H-5t$_{\text{sphing}}$), 5.30 (d, 1H, H-5c$_{\text{sphing}}$), 4.74 (d, overlapped with HOD, 1H, H-1$_{\text{GalNAc}}$), 4.52 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{\text{Gal}}$), 4.49 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{\text{Glc}}$), 4.32 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 7.0 Hz, H-3$_{\text{sphing}}$), 4.23 (ddd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 7.0 Hz, $J_{2,1b}$ = 3.9 Hz, H-2$_{\text{sphing}}$), 4.14 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 2.6 Hz, H-3$_{\text{Gal}}$), 4.12-4.10 (m, 2H, H-1a$_{\text{sphing}}$, H-4$_{\text{Gal}}$), 3.99 (d, 1H, $J_{6a,6b}$ = 12.1 Hz, H-6a$_{\text{Glc}}$), 3.93-3.86 (m, 4H, H-4$_{\text{GalNAc}}$, H-2$_{\text{GalNAc}}$, H-1b$_{\text{sphing}}$, H-9a$_{\text{Neu}}$), 3.83-3.59 (m, 16H, H-5$_{\text{Neu}}$, H-6b$_{\text{Glc}}$, H-4$_{\text{Neu}}$, H-5$_{\text{Gal}}$, H-6a$_{\text{Gal}}$, H-6b$_{\text{Gal}}$, H-5$_{\text{GalNAc}}$, H-6a$_{\text{GalNAc}}$, H-6b$_{\text{GalNAc}}$, H-8$_{\text{Neu}}$, H-3$_{\text{GalNAc}}$, H-3$_{\text{Glc}}$, H-9b$_{\text{Neu}}$, H-4$_{\text{Glc}}$, H-7$_{\text{Neu}}$, H-5$_{\text{Glc}}$), 3.49 (d, 1H, $J_{6,5}$ = 10.1 Hz, H-6$_{\text{Neu}}$), 3.37-3.31 (m, 2H, H-2$_{\text{Gal}}$, H-2$_{\text{Glc}}$), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e$_{\text{Neu}}$), 2.03, 2.02 (2 × s, 2 × 3H, 2 × C(O)$\underline{CH_3}$), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 11.8 Hz, H-3a$_{\text{Neu}}$); ^{13}C NMR (125 MHz, D_2O) : δ_C 175.9, 175.7, 174.9, 159.8, 136.4, 119.4, 116.7 (q, $J_{C,F}$ = 285 Hz), 103.7, 103.5, 103.0, 102.5, 79.5, 78.0, 75.7, 75.6, 75.2, 75.1, 74.9, 74.0, 73.5, 73.1, 72.5, 72.2, 70.9, 69.6, 68.9, 65.1, 63.7, 62.1, 61.5, 61.0, 54.8, 53.2, 52.5, 49.8, 37.9, 23.6, 22.9. ESI HRMS calc'd for $C_{38}H_{59}N_3O_{26}F_3$: 1030.3344. Found: 1030.3356.

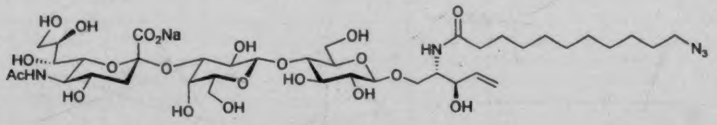
Procedure (A) for deprotection and acylation of sphingosine amine using succinimide esters.

Oligosaccharide **2** or **3** (5-40 μmol) was dissolved in dry methanol (2.0 mL). Sodium methoxide (~3.0 eq) was added and the mixture, stirred for 3 days at room temperature until completion of the reaction. The pH of the reaction mixture was adjusted to 9.0 using 0.5 M HCl and methanol was evaporated. The compound was purified by HPLC (Hamilton semi-preparative PRP-1 column) using a $MeOH/H_2O$ gradient. A portion of the amine (5-15 μmol), the acylating agent (2.0-5.0 eq) and 4-*N*,*N*-dimethylaminopyridine (1.0-3.0 eq) were dissolved in a 9:1 pyridine-water mixture (1.0 mL). The reaction mixture was stirred overnight at 40° C and concentrated. The residue was redissolved in a solution of 5% $NaHCO_3/H_2O$ and applied to a C18 Sep-Pak cartridge. Gradient elution ($MeOH-H_2O$) afforded the target compound which was further purified by size exclusion chromatography (Biogel P-4 resin; 50 cm × 2 cm) using 5% $MeOH-H_2O$ as an eluent.

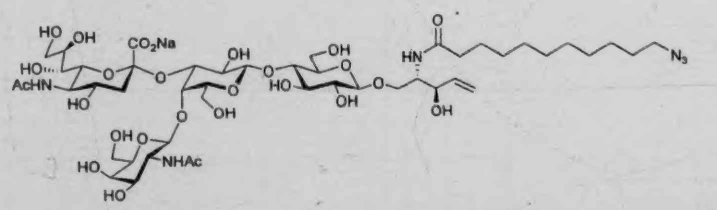


***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-octadecan-amido-pent-4-ene-1,3-diol (13).** Compound **13** was prepared using Procedure (A). Trisaccharide **2** (30.3 mg; 35.6 μmol) was deprotected and a portion of the amine (6.37 mg; 8.44 μmol) was acylated with *N*-(octadecanoyloxy)-succinimide (7.43 mg; 19.4 μmol; 2.3 eq). Standard work-up and purification as described in Procedure (A) afforded **13** (4.70 mg; 4.60 μmol; 55%): $[\alpha]_D$ +6.4° (*c* 0.34, H_2O). ^1H NMR (600 MHz, CD_3OD): δ_H 5.86 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 6.7 Hz, H-4_{sphing}), 5.26 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.5 Hz, $J_{5t,3}$ = 1.5 Hz, H-$5t_{sphing}$), 5.13 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,4}$ = 1.5 Hz, $J_{5c,3}$ = 1.5 Hz, H-$5c_{sphing}$), 4.42 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Gal}), 4.29 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Glc}), 4.17-4.13 (m, 2H, H-$1a_{sphing}$, H-3_{sphing}), 4.04 (dd, 1H, $J_{3,2}$ = 9.7 Hz, $J_{3,4}$ = 3.2 Hz, H-3_{Gal}), 4.01 (m, 1H, H-2_{sphing}), 3.93-3.82 (m, 5H, H-4_{Gal}, H-$6a_{Glc}$, H-$6a_{Gal}$, H-$6b_{Gal}$, H-6_{Neu}), 3.76 (dd, 1H, $J_{9a,9b}$ = 11.7 Hz, $J_{9a,8}$ = 7.7 Hz, H-$9a_{Neu}$), 3.71-3.69 (m, 2H, H-4_{Neu}, H-5_{Gal}), 3.65 (dd, 1H, $J_{9b,9a}$ = 11.7 Hz, $J_{9a,8}$ = 4.2 Hz, H-$9b_{Neu}$), 3.61-

3.52 (m, 7H, H-5$_{\text{Neu}}$, H-5$_{\text{Glc}}$, H-1b$_{\text{sphing}}$, H-8$_{\text{Neu}}$, H-2$_{\text{Gal}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$), 3.47 (dd, 1H, $J_{6b,6a}$ = 9.0 Hz, $J_{6b,5}$ = 1.8 Hz, H-6b$_{\text{Glc}}$), 3.42 (m, 1H, H-7$_{\text{Neu}}$), 3.27 (dd, 1H, $J_{2,3}$ = 7.8 Hz, $J_{2,1}$ = 7.8 Hz, H-2$_{\text{Glc}}$) 2.86 (m, 1H, H-3e$_{\text{Neu}}$) 2.18 (t, 2H, J = 7.5 Hz, $\underline{CH_2}C(O)$), 2.00 (s, 3H, $C(O)\underline{CH_3}$), 1.72 (m, 1H, H-3a$_{\text{Neu}}$), 1.59-1.56 (m, 2H, $\underline{CH_2}CH_2C(O)$), 1.30-1.28 (m, 28H, alkane $\underline{CH_2}$), 0.89 (t, J = 7.0 Hz, CH_3); ^{13}C NMR (125 MHz, CD_3OD): δ_C 176.2, 175.5, 174.9, 139.6, 116.9, 116.1, 105.1, 104.5, 101.1, 80.9, 77.7, 77.1, 76.5, 76.3, 75.0, 74.8, 73.3, 73.0, 70.9, 70.2, 69.71, 69.70, 69.4, 69.0, 64.7, 64.2, 62.8, 61.88, 61.86, 54.7, 54.00, 53.98, 49.01, 48.8, 42.1, 37.2, 33.1, 30.49, 30.46, 30.40, 27.1, 22.6, 14.4. ESI HRMS calc'd for $C_{46}H_{81}N_2O_{21}Na_2$: 1043.5127. Found: 1043.5124.

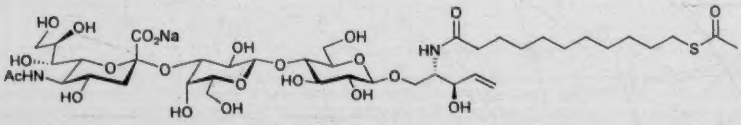


O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(11-azidoundecan-amido)-pent-4-ene-1,3-diol (14). Compound **14** was synthesized according to Procedure (A). Trisaccharide **2** (30.3 mg; 35.6 μmol) was deprotected and a portion of the amine (11.9 mg; 15.8 μmol) was acylated with *N*-(11-azidoundecanoyloxy)-succinimide (15.3 mg; 47.2 μmol; 3.0 eq). Standard work-up and purification as described in Procedure (A) yielded **14** (8.4 mg; 8.8 μmol; 78%): $[\alpha]_D$ +2.0° (c 0.45, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.83 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 6.9 Hz, H-4$_{\text{sphing}}$), 5.31 (ddd, 1H, $J_{5t,4}$ = 17.3 Hz, $J_{5t,5c}$ = 1.2 Hz, $J_{5t,3}$ = 1.2 Hz, H-5t$_{\text{sphing}}$), 5.26 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.2 Hz, $J_{5c,3}$ = 1.0 Hz, H-5b$_{\text{sphing}}$), 4.50 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{\text{Gal}}$), 4.45 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{\text{Glc}}$), 4.20 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 6.9 Hz, H-3$_{\text{sphing}}$), 4.11-4.04 (m, 3H, H-3$_{\text{Gal}}$, H-2$_{\text{sphing}}$, H-1a$_{\text{sphing}}$), 4.00 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.2 Hz, H-6a$_{\text{Glc}}$), 3.95 (d, 1H, $J_{4,3}$ = 3.1 Hz, H-4$_{\text{Gal}}$), 3.90-3.78 (m, 4H, H-8$_{\text{Neu}}$, H-9$_{\text{Neu}}$, H-6b$_{\text{Glc}}$, H-5$_{\text{Neu}}$), 3.76 (m, 1H, H-1b$_{\text{sphing}}$), 3.73-3.66 (m, 2H, H-5$_{\text{Gal}}$, H-4$_{\text{Neu}}$), 3.65-3.61 (m, 5H, H-6a$_{\text{Gal}}$, H-6b$_{\text{Gal}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$, H-9b$_{\text{Neu}}$), 3.60-3.55 (m, 4H, H-5$_{\text{Glc}}$, H-6$_{\text{Neu}}$, H-7$_{\text{Neu}}$, H-2$_{\text{Gal}}$), 3.35-3.29 (m, 3H, H-2$_{\text{Glc}}$, $\underline{CH_2}N_3$), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.7 Hz, H-3e$_{\text{Neu}}$), 2.25 (t, 2H, J = 7.3 Hz, $\underline{CH_2}C(O)$), 2.03 (s, 3H, C(O)$\underline{CH_3}$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-3a_{Neu}), 1.61-1.56 (m, 4H, $\underline{CH_2}CH_2C(O)$, $\underline{CH_2}CH_2N_3$), 1.38-1.29 (m, 12H, alkane $\underline{CH_2}$); ^{13}C NMR (125 MHz, D_2O): δ_C 178.2, 175.9, 174.7, 137.2, 119.0, 103.6, 103.3, 100.7, 79.4, 76.4, 76.1, 75.7, 75.1, 73.8, 73.7, 72.7, 72.6, 70.3, 69.6, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 53.9, 52.6, 52.2, 40.5, 36.8, 36.7, 29.4 (2C), 29.2, 29.1, 29.0, 28.8, 26.2, 22.9. ESI HRMS calc'd for $C_{39}H_{67}N_5O_2Na$: 964.4226. Found: 964.4223.

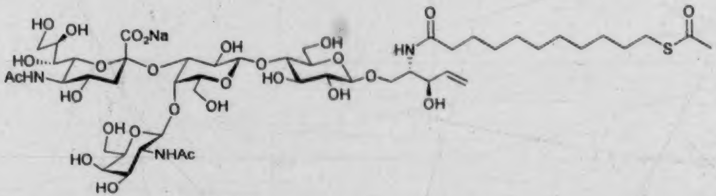


O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-O-β-D-galactopyranosyl-(1→4)]-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(11-azidoundecan-amido)-pent-4-ene-1,3-diol (15). Compound **15** was prepared using Procedure (A). Tetrasaccharide **3** was deprotected and a portion of the amine (4.8 mg; 5.0 μmol) was acylated with *N*-(11-azidoundecanoyloxy)-succinimide (2.4 mg; 7.4 μmol; 1.5 eq). Work-up and purification as described above afforded **15** (4.5 mg; 3.8 μmol; 76%): $[\alpha]_D$ +6.4° (*c* 0.45, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.33 (m, 1H, H-5t_{sphing}), 5.27 (m, 1H, H-5c_{sphing}), 4.74 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1_{GalNAc}), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Gal}), 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.22 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3_{sphing}), 4.15-4.06 (m, 4H, H-3_{Gal}, H-2_{sphing}, H-1a_{sphing}, H-4_{Gal}), 3.99 (d, 1H, $J_{6a,6b}$ = 12.1 Hz, H-6a_{Glc}), 3.92-3.90 (m, 2H, H-4_{GalNAc}, H-2_{GalNAc}), 3.87 (dd, 1H, $J_{9a,9b}$ = 11.0 Hz, $J_{9a,8}$ = 1.1 Hz, H-9a_{Neu}), 3.83-3.58 (m, 17H, H-5_{Neu}, H-6b_{Glc}, H-1b_{sphing}, H-4_{Neu}, H-5_{Gal}, H-6a_{Gal}, H-6b_{Gal}, H-5_{GalNAc}, H-6a_{GalNAc}, H-6b_{GalNAc}, H-8_{Neu}, H-3_{GalNAc}, H-3_{Glc}, H-9b_{Neu}, H-4_{Glc}, H-7_{Neu}, H-5_{Glc}), 3.48 (dd, 1H, $J_{6,5}$ = 9.9 Hz, $J_{6,7}$ = 1.9 Hz, H-6_{Neu}), 3.38-3.30 (m, 4H, H-2_{Gal}, H-2_{Glc}, $\underline{CH_2}N_3$), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.7 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e_{Neu}), 2.25 (t, 2H, J = 7.3 Hz, $\underline{CH_2}C(O)N$), 2.03, 2.01 (2 × s, 2 × 3H, 2 × C(O)$\underline{CH_3}$), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.7 Hz, $J_{3a,4}$ = 11.4 Hz, H-3a_{Neu}) 1.59-1.56 (m, 4H, $\underline{CH_2}CH_2N_3$, $\underline{CH_2}CH_2C(O)$), 1.38-1.27 (m, 12H, alkene CH_2);

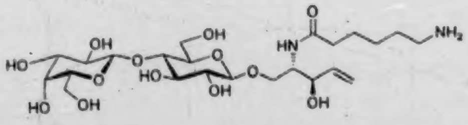
^{13}C NMR (125 MHz, D_2O) : δ_C 178.2, 175.9, 175.7, 174.9, 137.2, 136.4, 119.0, 103.6, 103.3, 102.5, 79.6, 78.0, 75.6 (2 C), 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.2, 70.9, 69.6, 68.9, 68.7, 64.6, 63.7, 62.0, 61.4, 53.9, 53.2, 52.5, 52.1, 42.8, 37.8, 36.8, 29.4, 29.3, 29.2, 29.1, 29.0, 28.8, 26.2, 26.1, 23.5, 22.9. ESI HRMS calc'd for $C_{47}H_{79}N_6O_{26}Na$: 1167.5020. Found: 1167.5021.



***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(11-thioacetylundecan-amido)-pent-4-ene-1,3-diol (16)**. Compound **16** was synthesized according to Procedure (A). Trisaccharide **2** (30.3 mg; 36.6 μmol) was deprotected and a portion of the amine (4.0 mg; 5.3 μmol) was acylated with (11-thioacetylundecanoyloxy)-succinimide[289] (3.8 mg; 10.6 μmol; 2.0 eq). Work-up and purification as described above yielded **16** (4.2 mg; 4.2 μmol; 80%): $[\alpha]_D$ +2.3° (*c* 0.42, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.84 (d, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{\text{sphing}}$), 5.32 (m, 1H, H-5t$_{\text{sphing}}$), 5.26 (m, 1H, H-5c$_{\text{sphing}}$), 4.51 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{\text{Gal}}$), 4.46 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{\text{Glc}}$), 4.21 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 7.0 Hz, H-3$_{\text{sphing}}$), 4.11-4.05 (m, 3H, H-3$_{\text{Gal}}$, H-2$_{\text{sphing}}$, H-1a$_{\text{sphing}}$), 3.99 (dd, 1H, $J_{6a,6b}$ = 12.1 Hz, $J_{6a,5}$ = 1.8 Hz, H-6a$_{\text{Glc}}$), 3.95 (d, 1H, $J_{4,3}$ = 3.0 Hz, H-4$_{\text{Gal}}$), 3.89-3.55 (m, 16H, H-8$_{\text{Neu}}$, H-9a$_{\text{Neu}}$, H-6b$_{\text{Glc}}$, H-5$_{\text{Neu}}$, H-1b$_{\text{sphing}}$, H-5$_{\text{Gal}}$, H-4$_{\text{Neu}}$, H-6a$_{\text{Gal}}$, H-6b$_{\text{Gal}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$, H-9b$_{\text{Neu}}$, H-5$_{\text{Glc}}$, H-6$_{\text{Neu}}$, H-7$_{\text{Neu}}$, H-2$_{\text{Gal}}$), 3.33 (m, 1H, H-2$_{\text{Glc}}$), 2.89 (t, 2H, J = 7.2 Hz, CH_2SAc), 2.76 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.6 Hz, H-3e$_{\text{Neu}}$), 2.36 (s, 3H, SC(O)CH_3), 2,25 (t, 2H, J = 7.2 Hz, CH_2C(O)N), 2.03 (s, 3H, C(O)CH_3), 1.80 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-3a$_{\text{Neu}}$), 1.60-1.55 (m, 4H, CH_2CH_2C(O)N, CH_2CH_2C(O)S), 1.37-1.28 (m, 12H, alkane CH_2); ^{13}C NMR (125 MHz, D_2O): δ_C 203.4, 178.2, 175.9, 174.7, 137.2, 119.0, 103.6, 103.4, 100.7, 79.4, 76.4, 76.1, 75.7, 75.1, 73.8, 73.7, 72.7, 72.6, 70.3, 69.6, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 53.9, 52.6, 40.5, 36.8, 31.0, 29.9, 29.41, 29.36, 29.3, 29.2, 29.1, 29.0, 28.7, 26.2, 22.9. ESI HRMS calc'd for $C_{41}H_{69}N_2O_{22}NaS$: 1019.3858. Found: 1019.3857.

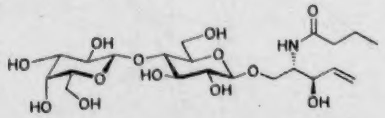


***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(11-thioacetylundecan-amido)-pent-4-ene-1,3-diol (17).** Compound **17** was prepared according to Procedure (A). Tetrasaccharide **3** (14.7 mg; 14.0 μmol) was deprotected and a portion of the amine (7.0 mg; 7.3 μmol) was acylated with (11-thioacetylundecanoyloxy)-succinimide[289] (5.20 mg; 14.6 μmol; 2.0 eq). Standard work-up and purification as described in Procedure (A) afforded **17** (8.3 mg; 6.9 μmol; 95%): $[\alpha]_D$ +15.4° (c 0.31, H_2O). ^1H NMR (500 MHz, D_2O): δ_H 5.83 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0Hz, H-4$_{sphing}$), 5.31 (m, 1H, H-5t$_{sphing}$), 5.25 (m, 1H, H-5c$_{sphing}$), 4.73 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1$_{GalNAc}$), 4.50 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Gal}$), 4.46 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.20 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3$_{sphing}$), 4.14-4.04 (m, 4H, H-3$_{Gal}$, H-2$_{sphing}$, H-1a$_{sphing}$, H-4$_{Gal}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 11.7 Hz, $J_{6a,5}$ = 1.5 Hz, H-6a$_{Glc}$), 3.93-3.86 (m, 3H, H-4$_{GalNAc}$, H-2$_{GalNAc}$, H-9a$_{Neu}$), 3.80-3.57 (m, 17H, H-5$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$, H-4$_{Neu}$, H-5$_{Gal}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{GalNAc}$, H-6a$_{GalNAc}$, H-6b$_{GalNAc}$, H-8$_{Neu}$, H-3$_{GalNAc}$, H-3$_{Glc}$, H-9b$_{Neu}$, H-4$_{Glc}$, H-7$_{Neu}$, H-5$_{Glc}$), 3.47 (dd, 1H, $J_{6,5}$ = 9.9 Hz, $J_{6,7}$ = 1.9 Hz, H-6$_{Neu}$), 3.37-3.31 (m, 2H, H-2$_{Gal}$, H-2$_{Glc}$), 2.88 (t, 2H, J = 7.2 Hz, $\underline{CH_2}$SAc), 2.65 (dd, 1H, $J_{3e,3a}$ = 12.8 Hz, $J_{3e,4}$ = 4.4 Hz, H-3e$_{Neu}$), 2.35 (s, 3H, SC(O)$\underline{CH_3}$), 2.24 (t, 2H, J = 7.3 Hz, $\underline{CH_2}$C(O)N), 2.03, 2.01 (2 × s, 2 × 3H, 2 × C(O)$\underline{CH_3}$), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.8 Hz, $J_{3a,4}$ = 11.5 Hz, H-3a$_{Neu}$) 1.59-1.56 (m, 4H, $\underline{CH_2}CH_2$SAc, $\underline{CH_2}CH_2$C(O)), 1.30-1.28 (m, 12H, alkane $\underline{CH_2}$); ^{13}C NMR (125 MHz, D_2O): δ_C 203.1, 178.2, 175.9, 175.7, 174.9, 137.5, 119.2, 103.6, 103.5, 102.7, 79.6, 78.0, 75.6 (2 C), 75.2, 75.1, 74.9, 73.9, 73.6, 73.1, 72.7, 72.2, 70.9, 69.5 (2C), 68.9, 68.6, 63.7, 62.1, 61.4, 61.0, 59.8, 53.9, 53.2, 52.5, 37.9, 36.8, 31.0, 29.9, 29.4, 29.3, 29.2, 29.0, 28.9, 28.6, 26.2, 23.5, 22.9. ESI HRMS calc'd for $C_{49}H_{82}N_3O_{27}SNa_2$: 1222.4652. Found: 1222.4448.



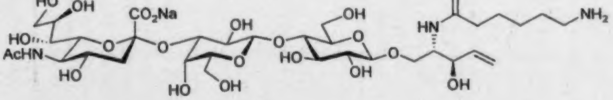
O-(β-D-Galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2S,3R)-2-(6-aminohexan-amido)-pent-4-ene-1,3-diol (18). Benzoylated lactosyl-azidosphingosine[153] (200 mg; 0.15 mmol) was dissolved in 90% aqueous pyridine (10.0 mL) and triphenylphosphine (100 mg; 0.38 mmol; 2.5 eq.) was added. After stirring for 8 hr, *p*-nitrophenyl 6-trifluoracetamidohexanoate (10 eq) was added, and stirring was continued overnight. The mixture was concentrated, applied to a short plug of silica gel in 2:1 hexanes-ethyl acetate, and eluted with a gradient of the same mixture (3:2→1:1). The material obtained was deprotected by stirring with a catalytic amount of sodium methoxide in methanol (5.0 mL) at 45 °C overnight. After cooling to room temperature, the solution was neutralized by addition of acetic acid, concentrated, suspended in water-methanol (95:5) and filtered. The solution was then applied to a Sep-Pak (C18; 5 g) which had been preconditioned with methanol and water containing ~2% ammonium hydroxide and eluted with a gradient of water and methanol containing the same. Concentration of the eluent afforded **18** (49.8 mg; 90.0 μmol; 58%). A portion of the material was purified further for analysis on a Hamilton PRP-1 semi-preparative HPLC column using a H_2O/MeOH gradient: $[\alpha]_D$ +4.9° (c 0.40, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.32 (m, 1H, H-5t$_{sphing}$), 5.27 (m, 1H, H-5c$_{sphing}$), 4.48 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.44 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{Gal}$), 4.22 (dd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 7.0 Hz, H-3$_{sphing}$), 4.11 (ddd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 6.4 Hz, $J_{2,1b}$ = 3.6 Hz, H-2$_{sphing}$), 4.06 (dd, 1H, $J_{1a,1b}$ = 10.6 Hz, $J_{1a,2}$ = 6.4 Hz, H-1a$_{sphing}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.2 Hz, H-6a$_{Glc}$), 3.93 (d, 1H, $J_{4,3}$ = 3.4 Hz, H-4$_{Gal}$), 3.86-3.69 (m, 5H, H-6b$_{Glc}$, H-1b$_{sphing}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{Gal}$), 3.67-3.62 (m, 3H, H-3$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$), 3.60 (m, 1H, H-5$_{Glc}$), 3.54 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 7.9 Hz, H-2$_{Gal}$), 3.33 (m, 1H, H-2$_{Glc}$), 2.98-2.95 (m, 2H, $\underline{CH_2}NH_2$), 2.36-2.30 (m, 2H,

$\underline{CH_2}C(O)N$), 1.68-1.58 (m, 4H, $\underline{CH_2}CH_2NH_2$, $\underline{CH_2}CH_2C(O)$)), 1.40-1.34 (m, 2H, CH_2); ^{13}C NMR (125 MHz, D_2O): δ_C 177.6, 137.2, 119.0, 103.8, 103.2, 79.4, 76.3, 75.7, 75.1, 73.7, 73.4, 72.8, 71.8, 69.5, 61.9, 60.9, 53.8, 40.2, 36.4, 27.5, 26.0, 25.6, 24.2. ESI HRMS calc'd for $C_{23}H_{43}N_2O_{13}$: 555.2760. Found: 555.2763.

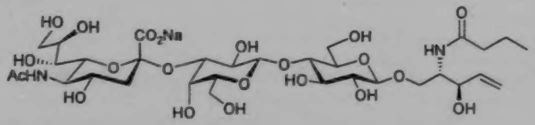


O-(β-D-Galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2S,3R)-2-butanamido-pent-4-ene-1,3-diol (19). Benzoylated lactosyl azidosphingosine[153] (3.6 g; 2.8 mmol) was dissolved in anhydrous methanol (40 mL) and treated with a catalytic amount of sodium methoxide. After stirring overnight, the mixture was neutralized with Rexyn 101 ion exchange resin. Removal of the resin by filtration and evaporation of the solvent yielded a white solid. Purification of the compound by reverse-phase column chromatography using water as an eluent afforded the deprotected compound (1.1 g; 2.3 mmol; 83%). A portion of the lactosyl azidosphingosine (151.5 mg; 324 μmol) was dissolved in a solution of pyridine/water/triethylamine (10:1:0.3, 11.3 mL). The solution was saturated with H_2S by bubbling the gas through the solution for 1 hr at 0°C. The mixture was stirred for 36 hr before the reaction was completed. The solvent was evaporated and the residue was dissolved in water. After addition of 200 μL of acetic acid, the mixture was applied to a Sep-Pak column (C18; 5 g). The salt obtained was eluted through Amberlite IRA-400 (Cl^-) resin preconditioned with 2 M NaOH to yield the free amine (124.7 mg; 282 μmol; 87%). A portion of the material (20.0 mg; 45.0 μmol) was dissolved in methanol (2.0 mL) and butyric anhydride (19 μL; 113 μmol; 2.5 eq) was added. After stirring for 2.5 hr the solution was concentrated and the residue obtained dissolved in water (1.5 mL). The mixture was applied to a C18 Sep-Pak cartridge and eluted with water to afford **19** (20.0 mg; 39.0 μmol; 86%): $[\alpha]_D$ +8.8° (c 0.15, H_2O). ^1H NMR (600 MHz, D_2O): δ_H 5.86 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.33 (m, 1H, H-$5t_{sphing}$), 5.27 (m, 1H, H-$5c_{sphing}$), 4.48 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.45 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 4.23 (dd, 1H, $J_{2,3}$ = 7.1 Hz, $J_{2,1a}$ = 7.1 Hz, H-

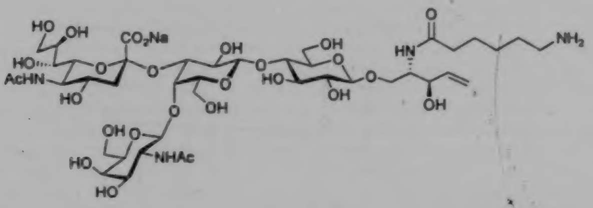
3$_{sphing}$), 4.11 (ddd, 1H, $J_{2,3}$ = 7.1 Hz, $J_{2,1a}$ = 6.7 Hz, $J_{2,1b}$ = 3.7 Hz, H-2$_{sphing}$), 4.06 (dd, 1H, $J_{1a,1b}$ = 10.5 Hz, $J_{1a,2}$ = 6.4 Hz, H-1a$_{sphing}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.1 Hz, H-6a$_{Glc}$), 3.93 (d, 1H, $J_{4,3}$ = 3.3 Hz, H-4$_{Gal}$), 3.83-3.71 (m, 5H, H-6b$_{Glc}$, H-1b$_{sphing}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{Gal}$), 3.68-3.62 (m, 3H, H-3$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$), 3.60 (m, 1H, H-5$_{Glc}$), 3.54 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 7.9 Hz, H-2$_{Gal}$), 3.35 (m, 1H, H-2$_{Glc}$) 2.23 (t, 2H, J = 7.4 Hz, $\underline{CH_2}$C(O)N), 1.62-1.58 (sextet, 2H, J = 7.4 Hz, $\underline{CH_2}CH_3$), 0.90 (t, 3H, J = 7.4 Hz, $\underline{CH_3}$); ^{13}C NMR (from HMQC/HMBC spectra): δ_C 178.2, 137.2, 119.0, 103.8, 103.2, 79.5, 79.4, 76.4, 75.4, 75.2, 73.5, 73.3, 72.8, 71.8, 69.6, 69.4, 62.0, 53.9, 38.8, 19.9, 13.7. ESI HRMS calc'd for $C_{21}H_{37}NO_{13}Na$: 534.2157. Found: 534.2151.



O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(6-aminohexan-amido)-pent-4-ene-1,3-diol (20). Compound **30** (15.3 mg, 15.9 μmol) was dissolved in dry degassed methanol (3.0 mL) and sodium methoxide (275 μL of 0.173 M solution; 47.6 μmol; 3.0 eq) was added. The reaction mixture was stirred at 40 °C for 36 hr. The pH of the reaction mixture was adjusted to 9.0 using 0.5 M HCl and the methanol was evaporated. The compound was purified by HPLC using a Hamilton PRP-1 semi-preparative column. Elution with a MeOH-H_2O gradient afforded **20** (10.0 mg; 11.5 μmol; 72%). Alternatively, the acceptor **18** (25 mg; 45 μmol; 9.0 mM in reaction) was combined with CMP-sialic acid (135 mg of crude mixture, estimated 1.3 eq.), 0.5 M $MnCl_2$ (100 μL; 10 mM in reaction), 0.5 M $MgCl_2$ (100 μL; 10 mM in reaction), 0.4 M dithiothreitol (37.5 μL; 3 mM in reaction), α-(2,3)-sialyltransferase (4.75 mL; ~4.75 U), and alkaline phosphatase (8 μL; 8U). The mixture was tumbled gently overnight and then centrifuged at 14,000 rpm for 30 min to remove cellular debris. The supernatant was applied directly to a size exclusion column (Biogel P-4; 50 × 2 cm). Elution with 5% aqueous methanol afforded **20** (31 mg; 36 μmol; 80%): $[\alpha]_D$ +2.7° (c 0.24, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.84 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.31 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.2 Hz, $J_{5t,3}$ = 1.2 Hz, H-5t$_{sphing}$), 5.25 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.2 Hz, $J_{5c,3}$ = 1.2 Hz, H-5c$_{sphing}$), 4.51 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Gal}$), 4.46 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Glc}$), 4.21 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 6.6 Hz, H-3$_{sphing}$), 4.12-4.08 (m, 2H, H-2$_{sphing}$, H-3$_{Gal}$), 4.04 (dd, 1H, $J_{1a,1b}$ = 10.5 Hz, $J_{1a,2}$ = 6.4 Hz, H-1a$_{sphing}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.2 Hz, H-6a$_{Glc}$), 3.94 (d, 1H, $J_{4,3}$ = 3.1 Hz, H-4$_{Gal}$), 3.90-3.73 (m, 5H, H-8$_{Neu}$, H-5$_{Neu}$, H-9a$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$), 3.71-3.67 (m, 3H, H-4$_{Neu}$, H-6a$_{Gal}$, H-6b$_{Gal}$), 3.66-3.61 (m, 5H, H-5$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$, H-9b$_{Neu}$, H-6$_{Neu}$), 3.59-3.55 (m, 3H, H-5$_{Glc}$, H-7$_{Neu}$, H-2$_{Gal}$), 3.32 (m, 1H, H-2$_{Glc}$), 2.97 (t, 2H, J = 7.6 Hz, $\underline{CH_2}NH_2$), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.2 Hz, $J_{3e,4}$ = 4.6 Hz, H-3e$_{Neu}$), 2.28 (t, 2H, J = 7.4 Hz, $\underline{CH_2}C(O)$), 2.03 (s, 3H, $C(O)\underline{CH_3}$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.2 Hz, $J_{3a,4}$ = 12.1 Hz, H-3a$_{Neu}$), 1.69-1.59 (m, 4H, $\underline{CH_2}CH_2NH_2$, $\underline{CH_2}CH_2C(O)$), 1.41-1.34 (m, 2H, $\underline{CH_2}$); ^{13}C NMR (125 MHz, D_2O) : δ_C 177.6, 175.9, 174.7, 137.2, 118.9, 103.6, 103.2, 100.7, 79.2, 76.4, 76.1, 75.6, 75.1, 73.8, 73.7, 72.8, 72.7, 70.2, 69.5, 69.2, 69.0, 68.4, 63.5, 61.9, 60.9, 53.8, 52.6, 40.6, 40.2, 36.4, 27.4, 26.0, 25.6, 22.9. ESI HRMS calc'd for $C_{34}H_{60}N_3O_{21}$: 846.3719. Found: 846.3718.

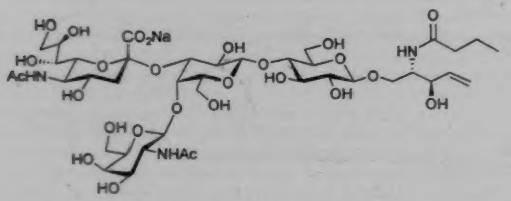


O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(butan-amido)-pent-4-ene-1,3-diol (21). Lactoside **19** (20.0 mg; 36.3 μmol) and crude CMP-Neu5Ac (~47.0 μmol; ~11.1 mM in reaction; ~1.2 eq) were combined as solids. $MnCl_2$ (100 μL of a 0.5 M solution; 10 mM in reaction), $MgCl_2$ (100 μL of a 0.5 M solution; 10 mM in reaction), dithiothreitol (32 μL of a 0.4 M solution; 2.5 mM in reaction), α-(2,3)-sialyltransferase (4.0 mL; ~ 4.0 U) and alkaline phosphatase (5 μL; ~5U) were then added sequentially and the mixture was tumbled gently overnight. When starting material had been consumed as judged by TLC ($CHCl_3$-MeOH-H_2O-AcOH; 10:7:1:2), the mixture was centrifuged at 14,000 rpm for 30 min and the supernatant was applied to a Sep-Pak (C18; 5 g) which was eluted with water (30 mL), followed by a gradient of water-methanol. The product obtained was further purified by HPLC (Kromasil C_{18}-silica semi-preparative column) using a gradient of water and methanol as eluents to afford **21** (24.9 mg; 31.0 μmol; 79%): $[\alpha]_D$ +2.4° (*c* 0.30, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (dddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.1 Hz, $J_{4,2}$ = 1.1Hz, H-4$_{\text{sphing}}$), 5.32 (m, 1H, H-5t$_{\text{sphing}}$), 5.27 (m, 1H, H-5c$_{\text{sphing}}$), 4.52 (dd, 1H, $J_{1,2}$ = 7.8 Hz, $J_{1,3}$ = 0.9 Hz, H-1$_{\text{Gal}}$), 4.47 (dd, 1H, $J_{1,2}$ = 7.9 Hz, $J_{1,3}$ = 0.9 Hz, H-1$_{\text{Glc}}$), 4.22 (ddd, 1H, $J_{3,4}$ = 7.1 Hz, $J_{3,2}$ = 7.1 Hz, $J_{3,1}$ = 0.9Hz, H-3$_{\text{sphing}}$), 4.12-4.05 (m, 3H, H-3$_{\text{Gal}}$, H-2$_{\text{sphing}}$, H-1a$_{\text{sphing}}$), 3.99 (d, 1H, $J_{6a,6b}$ = 11.4 Hz, $J_{6a,5}$ = 1.8 Hz, H-6a$_{\text{Glc}}$), 3.95 (d, 1H, $J_{4,3}$ = 2.7 Hz, H-4$_{\text{Gal}}$), 3.90-3.73 (m, 5H, H-8$_{\text{Neu}}$, H-5$_{\text{Neu}}$, H-9a$_{\text{Neu}}$, H-6b$_{\text{Glc}}$, H-1b$_{\text{sphing}}$), 3.71-3.62 (m, 8H, H-4$_{\text{Neu}}$, H-6a$_{\text{Gal}}$, H-6b$_{\text{Gal}}$, H-5$_{\text{Gal}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$, H-9b$_{\text{Neu}}$, H-6$_{\text{Neu}}$), 3.60-3.55 (m, 3H, H-5$_{\text{Glc}}$, H-7$_{\text{Neu}}$, H-2$_{\text{Gal}}$), 3.33 (m, 1H, H-2$_{\text{Glc}}$), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.6 Hz, H-3e$_{\text{Neu}}$), 2.22 (t, 2H, *J* = 7.1 Hz, $\underline{CH_2}C(O)$), 2.03 (s, 3H, $C(O)\underline{CH_3}$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.1 Hz, H-3a$_{\text{Neu}}$), 1.62-1.56 (m, 4H, $\underline{CH_2}CH_2C(O)$, $\underline{CH_2}CH_2CH_2C(O)$), 0.89 (t, 3H, *J* = 9.3 Hz, $\underline{CH_3}$); ^{13}C NMR (125 MHz, D_2O): δ_C 178.1, 175.9, 174.8, 137.1, 119.0, 103.6, 103.3, 100.7, 79.2, 76.4, 76.1, 75.6, 75.1, 73.8, 73.7, 72.74, 72.66, 70.3, 69.5, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 53.9, 52.6, 40.7, 38.7, 22.9, 19.9, 13.7. ESI HRMS calc'd for $C_{32}H_{53}N_2O_{21}$: 801.3134. Found: 801.3146.



***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(6-aminohexanamido)-pent-4-ene-1,3-diol (22).** Compound **31** (6.0 mg; 5.1 μmol) was dissolved in dry degassed methanol (1.5 mL) and sodium methoxide (89 μL of 0.17 M solution; 15.4 μmol; 3.0 eq) was added. The reaction mixture was stirred at 35° C for 36 hr. The methanol was evaporated, the residue obtained redissolved in water and the pH of the mixture was adjusted to 9.0 with 1N hydrochloric acid. The compound was purified by

HPLC using a Hamilton PRP-1 semi-preparative column. Elution with a MeOH-H_2O gradient afforded 22 (4.0 mg; 3.7 μmol; 73%). Alternatively, UDP-*N*-acetylglucosamine disodium salt (15 mg; 34 μmol; 1.2 eq.) was added to a Falcon tube containing **20** (23.4 mg; 28.0 μmol; 1.0 eq.). *N*-Acetylgalactosaminyl transferase (1.5 mL, ~1.5U) in 50 mM HEPES, pH 7.5 containing 10% glycerol (v/v) and UDP *N*-acetylglucosamine 4-epimerase (1.5 mL, ~1.5 U) in 0.2 M NaCl, 1.0 mM EDTA, 20mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 were added to the mixture, followed by 75 μL of 0.5M $MnCl_2$ and alkaline phosphatase (Roche Biosciences) (10 μL; 10 U). The mixture was tumbled overnight and then centrifuged for 30 min at 14,000 rpm. The supernatant was applied to a Sep-Pak (C18; 5g), washed with water and eluted with a water-methanol gradient. The residue thus obtained was applied to a Biogel P-4 column (50 cm × 2 cm) and eluted with 5% MeOH/H_2O to yield **22** (23 mg; 22 μmol; 79%): $[\alpha]_D$ +27.1° (*c* 0.24, H_2O). ^1H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{\text{sphing}}$), 5.32 (m, 1H, H-5t$_{\text{sphing}}$), 5.27 (m, 1H, H-5c$_{\text{sphing}}$), 4.73 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1$_{\text{GalNAc}}$), 4.52 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{\text{Gal}}$), 4.48 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{\text{Glc}}$), 4.23 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3$_{\text{sphing}}$), 4.14 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 3.0 Hz, H-3$_{\text{Gal}}$), 4.12-4.09 (m, 2H, H-4$_{\text{Gal}}$, H-2$_{\text{sphing}}$), 4.06 (dd, 1H, $J_{1a,1b}$ = 10.4 Hz, $J_{1a,2}$ = 6.2 Hz, H-1a$_{\text{sphing}}$), 3.99 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 1.9 Hz, H-6a$_{\text{Glc}}$), 3.93-3.90 (m, 2H, H-4$_{\text{GalNAc}}$, H-2$_{\text{GalNAc}}$) 3.87 (dd, 1H, $J_{9a,9b}$ = 12.0 Hz, $J_{9a,8}$ = 2.2 Hz, H-9a$_{\text{Neu}}$), 3.83-3.58 (m, 17H, H-5$_{\text{Neu}}$, H-6b$_{\text{Glc}}$, H-1b$_{\text{sphing}}$, H-4$_{\text{Neu}}$, H-5$_{\text{Gal}}$, H-6a$_{\text{Gal}}$, H-6b$_{\text{Gal}}$, H-5$_{\text{GalNAc}}$, H-6a$_{\text{GalNAc}}$, H-6b$_{\text{GalNAc}}$, H-8$_{\text{Neu}}$, H-3$_{\text{GalNAc}}$, H-3$_{\text{Glc}}$, H-9b$_{\text{Neu}}$, H-4$_{\text{Glc}}$, H-7$_{\text{Neu}}$, H-5$_{\text{Glc}}$), 3.49 (dd, 1H, $J_{6,5}$ = 10.1 Hz, $J_{6,7}$ = 2.1 Hz, H-6$_{\text{Neu}}$), 3.36 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 7.8 Hz, H-2$_{\text{Gal}}$), 3.32 (dd, 1H, $J_{2,3}$ = 9.2 Hz, $J_{2,1}$ = 8.1 Hz, H-2$_{\text{Glc}}$), 2.98 (t, 2H, *J* = 7.6 Hz, $\underline{CH_2}NH_2$), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.3 Hz, H-3e$_{\text{Neu}}$), 2.28 (t, 2H, *J* = 7.3 Hz, $\underline{CH_2}C(O)$), 2.03, 2.01 (2 × s, 2 × 3H, 2 × C(O)$\underline{CH_3}$), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-3a$_{\text{Neu}}$) 1.69-1.60 (m, 4H, $\underline{CH_2}CH_2NH_2$, $\underline{CH_2}CH_2C(O)$), 1.40-1.36 (m, 2H, CH_2); ^{13}C NMR (125 MHz, D_2O) : δ_C 177.6, 175.9, 175.7, 174.9, 137.2, 118.9, 103.6, 103.5, 103.2, 102.5, 79.5, 78.0, 75.6 (2 C), 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.1, 70.9, 69.6, 69.5, 68.9, 68.7, 63.7, 62.0, 61.5, 61.0, 53.8, 53.2, 52.5, 40.2, 37.9, 36.4, 27.4, 26.0, 25.6, 23.5, 22.9. ESI HRMS calc'd for $C_{42}H_{73}N_4O_{26}$: 1049.4513. Found: 1049.4513.



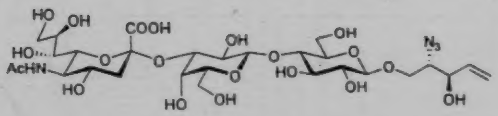
O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid-(2→3)-[2-acetamido-2-deoxy-*O*-(β-D-galactopyranosyl)-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(butan-amido)-pent-4-ene-1,3-diol (23). UDP-*N*-acetylglucosamine disodium salt (15 mg; 18 μmol; 1.2 eq.) was added to a Falcon tube containing **21** (12 mg; 15 μmol; 1.0 eq). Freshly prepared β-(1,4)-*N*-acetylgalactosaminyl transferase (2.0 mL; ~1.6 U) in 50 mM HEPES, pH 7.5 containing 10% glycerol (v/v) and UDP *N*-acetylglucosamine 4-epimerase (2.0 mL; ~2.0 U) in 0.2 M NaCl, 1.0 mM EDTA, 20 mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 were added to the mixture, followed by 4.0 μL of 0.5M $MnCl_2$ (0.5 mM in reaction) and 8 μL (~8 U) of alkaline phosphatase (Roche Biosciences). The mixture was tumbled gently overnight and then centrifuged for 20 min at 14,000 rpm. The supernatant was applied to a C18 Sep-Pak cartridge (C18; 5g), washed with water and eluted with a water-methanol gradient. The residue thus obtained was applied to a Biogel P-4 column in 5% aqueous methanol (50 cm × 2 cm) to yield **23** (13 mg; 13 μmol; 83%): $[\alpha]_D$ +14.8° (*c* 0.73, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.32 (m, 1H, H-$5t_{sphing}$), 5.26 (m, 1H, H-$5c_{sphing}$), 4.73 (d, overlapped with HOD, 1H, H-1_{GalNAc}), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Gal}), 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.22 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3_{sphing}), 4.14 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 3.0 Hz, H-3_{Gal}), 4.11-4.05 (m, 3H, H-4_{Gal}, H-2_{sphing}, H-$1a_{sphing}$), 3.99 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 1.9 Hz, H-$6a_{Glc}$), 3.92-3.89 (m, 2H, H-4_{GalNAc}, H-2_{GalNAc}) 3.87-3.58 (m, 18H, H-$9a_{Neu}$, H-5_{Neu}, H-$6b_{Glc}$, H-$1b_{sphing}$, H-4_{Neu}, H-5_{Gal}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{GalNAc}, H-$6a_{GalNAc}$, H-$6b_{GalNAc}$, H-8_{Neu}, H-3_{GalNAc}, H-3_{Glc}, H-$9b_{Neu}$, H-4_{Glc}, H-7_{Neu}, H-5_{Glc}), 3.48 (dd, 1H, $J_{6,5}$ = 10.1 Hz, $J_{6,7}$ = 1.8 Hz, H-6_{Neu}), 3.37-3.31 (m, 2H, H-2_{Gal}, H-2_{Glc}), 2.65 (dd, 1H, $J_{3e,3a}$ = 12.6 Hz, $J_{3e,4}$ = 4.5 Hz, H-$3e_{Neu}$), 2.22 (t, 2H, J = 7.3 Hz, $\underline{CH_2}C(O)$), 2.02, 2.01 (2 × s, 2 × 3H, 2 × $C(O)\underline{CH_3}$), 1.91 (dd, 1H, $J_{3a,3e}$ = 12.2 Hz, $J_{3a,4}$ = 12.2 Hz, H-$3a_{Neu}$) 1.61-1.55 (m, 4H, $\underline{CH_2}CH_2CH_2C(O)$, $\underline{CH_2}CH_2C(O)$), 0.89 (t, 3H, $J = 7.3$ Hz, CH_3); ^{13}C NMR (125 MHz, D_2O): δ_C 178.0 (2C), 175.9, 174.9, 137.1, 119.0, 103.6, 103.5, 103.2, 102.5, 79.5, 78.0, 75.60, 75.59, 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.2, 70.9, 69.6, 69.5, 68.9, 68.7, 63.7, 62.0, 61.4, 61.0, 53.9, 53.2, 52.5, 38.7, 37.9, 23.5, 22.9, 19.9, 13.7. ESI HRMS calc'd for $C_{40}H_{66}N_3O_{26}$: 1004.3940 Found: 1004.3940.

General procedure for the production of oligosaccharides in high-cell density culture

High-cell density cultures were carried out in 2-liter bioreactors containing 700 mL of mineral culture medium as previously described.[70] The mineral medium had the following composition: glucose (12.5 g/L), ammonium phosphate dibasic (7.0 g/L), potassium phosphate dibasic (7.0 g/L), citric acid (0.5 g/L), potassium hydroxide (2.5 g/L), sodium hydroxide (1.0 g/L), magnesium sulfate heptahydrate (1.0 g/L), thiamine hydrochloride (4.5 mg/L), and trace mineral solution (7.5 ml/L). Magnesium sulfate and glucose were added from concentrated solutions that were autoclaved separately. Thiamine was sterilized by filtration. The trace mineral solution contained: nitriloacetate (70 mM, pH 6.5), ferric citrate (7.5 g/L), manganese chloride tetrahydrate (1.3 g/L), cobalt (II) chloride hexahydrate (0.21 g/L) copper (II) chloride dihydrate (0.13 g/L), boric acid (0.25 g/L), zinc sulfate heptahydrate (1.2 g/L) and sodium molybdate dihydrate (0.15 g/L). Antibiotics were used to ensure maintenance of the different plasmids and were prepared in concentrated stock solutions. Their final concentrations were 50 mg/L for ampicilin and tetracycline and 25 mg/L for chloramphenicol. The temperature was maintained at 34 °C and the pH regulated to 6.8. The culture was initiated by inoculation of the bioreactor with 15 mL of a preculture on LB medium which had been incubated on a rotary shaker at 28 °C for approximately 5 h. The high-cell density culture strategy included two phases: an exponential growth phase which started with inoculation of the bioreactor and lasted until exhaustion of the carbon substrate (glucose) initially added to the medium and a fed-batch phase with a substrate feeding rate of 1.35 g/L/h. The feeding solution contained: glycerol (200 g/L), magnesium sulfate heptahydrate (5 g/L), and trace mineral solution (10 mL/L). The acceptor (truncated lactosyl sphingosine) was added at the beginning of the fed-batch phase at the same time as the inducer (IPTG, 50 mg/L) of the recombinant genes under the control of the Lac repressor. Culture samples were prepared and analyzed by thin layer chromatography as previously described.[201] Thin layer chromatography was carried out on silica gel plates and the oligosaccharides were eluted with *n*-butanol/acetic acid/water (2:1:1). The sugars were detected by dipping the plate in orcinol/sulphuric acid reagent and heating.

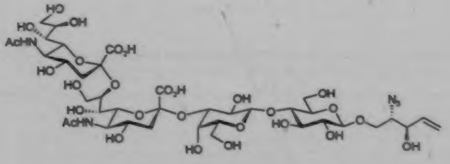
Purification of oligosaccharides produced by engineered bacteria

At the end of the fermentation period, bacterial cells were recovered by centrifugation (5000 rpm, 25 min) and the supernatant was designated 'extracellular fraction'. The cell pellet was resuspended in distilled water (approximately 700 mL) and the cells were permeabilized by autoclaving at 100 °C for 50 min. The mixture was again centrifuged (5000 rpm, 25 min) and the oligosaccharide-containing supernatant was recovered and designated 'intracellular fraction'. This fraction was treated with Amberlite IR-120 acidic resin until the pH reached 3.0 to precipitate the proteins. The mixture was decanted to recover the resin and the suspension obtained centrifuged (5000 rpm, 25 min). The supernatant was recovered and passed through a small column (2 cm × 1 cm) of Dowex 1 Cl^- resin. After this treatment, the pH of the solution was adjusted to 5.0 using Dowex 66 OH^- resin. The product was then purified by ion-exchange chromatography using a Dowex 1 HCO_3^- column (5 cm × 40 cm) and the negatively charged oligosaccharides, eluted using a linear gradient of bicarbonate buffer (0.0 M $\rightarrow$ 1.0 M, pH = 8.2, flow rate = 6 mL/min). The oligosaccharide containing fractions were then treated with Amberlite IR-120 acidic resin and degassed to remove bicarbonate from solution. After removal of the resin by filtration, the pH of the solution was adjusted to 5.0 with a 0.1 M NaOH solution and the solution lyophilized. The crude product obtained was further purifird by size exclusion chromatography on a HW40 column (5cm × 1 m) at 60 °C using 0.1 M bicarbonate buffer, pH 8.2 as eluent. The oligosaccharide-containing fractions were again treated, as described above, with Amberlite IR-120 resin to remove bicarbonate from solution. After neutralization and lyophilization, pure products were obtained.



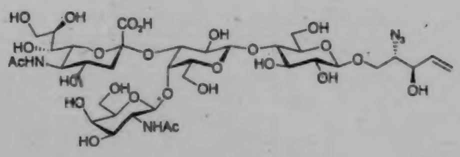
O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-azido-pent-4-ene-1,3-diol (24).

Compound **24** was prepared with bacterial strain DC7 using the general procedure for the production of oligosaccharide in high-cell density culture. Lactoside **11** (500 mg) was fed to the bacterial culture and incubated for 4 h. After isolation and purification of the oligosaccharide following the general procedure described above, GM_3 derivative was obtained (370 mg). GM_3 derivative **24** could also be produced with strain NF20. Under the same culture conditions, 83 mg of product were obtained: $[\alpha]_D$ –2.89° (*c* 0.61, H_2O). ^1H NMR (600 MHz, D_2O): δ_H 5.92 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.2 Hz, H-4_{sphing}), 5.38 (ddd, 1H, $J_{5t,4}$ = 17.3 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 1.2 Hz, H-$5t_{sphing}$), 5.33 (ddd, 1H, $J_{5c,4}$ = 10.5 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 1.2 Hz, H-$5c_{sphing}$), 4.53 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Gal}), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.36-4.34 (m, 1H, H-3_{sphing}), 4.11 (dd, 1H, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 3.2 Hz, H-3_{Gal}), 3.99 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.2 Hz, H-$6a_{Glc}$), 3.96 (d, 1H, $J_{4,3}$ = 3.2 Hz, H-4_{Gal}), 3.92-3.81 (m, 10H, H-$1a_{sphing}$, H-$1b_{sphing}$, H-8_{Neu}, H-$9a_{Neu}$, H-2_{sphing}, H-$6b_{Glc}$, H-$6a_{Gal}$, H-$9b_{Neu}$, H-7_{Neu}, H-5_{Neu}), 3.76-3.73 (m, 2H, H-$6b_{Gal}$, H-5_{Gal}), 3.71-3.62 (m, 5H, H-4_{Neu}, H-3_{Glc}, H-6_{Neu}, H-3_{Gal}, H-5_{Neu}), 3.60-3.56 (m, 3H, H-4_{Glc}, H-5_{Glc}, H-2_{Gal}), 3.35 (dd, 1H, $J_{2,1}$ = 8.5 Hz, $J_{2,3}$ = 8.5 Hz, H-2_{Glc}), 2.76 (dd, 1H, $J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 4.6 Hz, H-$3e_{Neu}$), 2.03 (s, 3H, $-C(O)CH_3$), 1.79 (dd, 1H, $J_{3a,3e} \approx J_{3a,4} \approx$ 12.1 Hz, H-$3a_{Neu}$); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 174.7, 136.0, 119.6, 103.0, 100.7, 79.1, 76.4, 76.1, 75.7, 75.2, 73.8, 73.6, 73.1, 72.7, 70.3, 69.6, 69.2, 69.0, 68.4, 65.8, 63.5, 61.9, 60.9, 52.6, 40.6, 22.9. ESI HRMS calc'd for $C_{28}H_{45}N_4O_{20}$: 757.2632. Found: 757.2639.



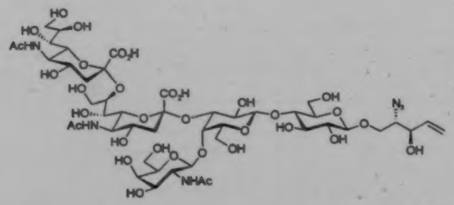
***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-azido-pent-4-ene-1,3-diol (25).**

Compound **25** was prepared with bacterial strain NF20 using the general procedure for the production of oligosaccharide in high-cell density culture. Lactoside **11** (500 mg) was fed to the bacterial culture and incubated for 4 h. After isolation and purification of the oligosaccharide following the general procedure described above, the GD_3 derivative **25** was obtained (272 mg): $[\alpha]_D$ –0.09° (*c* 0.43, H_2O). Selected ^{1}H NMR (600 MHz, D_2O): δ_H 5.92 (ddd, 1H, $J_{4,5t} = 17.3$ Hz, $J_{4,5c} = 10.4$ Hz, $J_{4,3} = 6.8$ Hz, H-4$_{\text{sphing}}$), 5.38 (ddd, 1H, $J_{5t,4} = 17.4$ Hz, $J_{5t,5c} \approx J_{5t,3} \approx 1.1$ Hz, H-5t$_{\text{sphing}}$), 5.33 (ddd, 1H, $J_{5c,4} = 9.9$ Hz, $J_{5c,5t} \approx J_{5c,3} \approx 1.0$ Hz, H-5c$_{\text{sphing}}$), 4.52 (d, 1H, $J_{1,2} = 8.0$ Hz, H-1$_{\text{Gal}}$), 4.50 (d, 1H, $J_{1,2} = 8.1$ Hz, H-1$_{\text{Glc}}$), 4.36-4.34 (m, 1H, H-3$_{\text{sphing}}$), 4.17 (dd, 1H, $J_{9a,9b} = 12.0$ Hz, $J_{9a,8} = 3.6$ Hz, H-9a$_{\text{Neu2}}$), 4.14-4.12 (m, 1H, H-8$_{\text{Neu2}}$), 4.08 (dd, 1H, $J_{3,2} = 9.9$ Hz, $J_{3,4} = 3.1$ Hz, H-3$_{\text{Gal}}$), 4.00 (dd, 1H, $J_{6a,6b} = 12.0$ Hz, $J_{6a,5} = 2.1$ Hz, H-6a$_{\text{Glc}}$), 3.96 (d, 1H, $J_{4,3} = 3.1$ Hz, H-4$_{\text{Glc}}$), 3.92-3.80 (m, H-1a$_{\text{sphing}}$, H-1b$_{\text{sphing}}$, H-2$_{\text{sphing}}$, H-6b$_{\text{Glc}}$, H-7$_{\text{Neu2}}$), 3.76-3.55 (m, H-5$_{\text{NeuB}}$, H-5$_{\text{NeuA}}$, H-5$_{\text{Gal}}$, H-9b$_{\text{Neu2}}$, H-4$_{\text{NeuA}}$, H-4$_{\text{NeuB}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$, H-6$_{\text{NeuA}}$, H-6$_{\text{NeuB}}$, H-5$_{\text{Glc}}$, H-2$_{\text{Gal}}$), 3.35 (dd, 1H, $J_{2,1} = 8.6$ Hz, $J_{2,3} = 8.6$ Hz, H-2$_{\text{Glc}}$), 2.77 (dd, 1H, $J_{3e,3a} = 12.3$ Hz, $J_{3e,4} = 4.6$ Hz, H-3e$_{\text{NeuA}}$), 2.67 (dd, 1H, $J_{3e,3a} = 12.3$ Hz, $J_{3e,4} = 4.3$ Hz, H-3e$_{\text{NeuB}}$), 2.06, 2.02 (2 × s, 2 × 3H, 2 × C(O)$\underline{CH_3}$), 1.73 (dd, 1H, $J_{3a,3e} \approx J_{3a,3e} \approx J_{3a,4} \approx J_{3a,4} \approx 12.1$ Hz, H-3a$_{\text{Neu1}}$, H-3a$_{\text{Neu2}}$); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 174.4, 174.2, 162.9, 136.0, 119.6, 103.6, 103.3, 101.5, 101.1, 79.1, 78.9, 76.4, 76.1, 75.8, 75.2, 74.9, 73.7, 73.6, 73.1, 72.6, 70.3, 70.2, 69.5, 69.4, 69.1, 68.8, 68.4, 65.8, 64.2, 63.5, 62.5, 62.0, 60.9, 53.2, 52.7, 41.4, 40.6, 23.2, 23.0. ESI HRMS calc'd for $C_{39}H_{61}N_5O_{28}$: 1047.3514. Found: 523.6756.



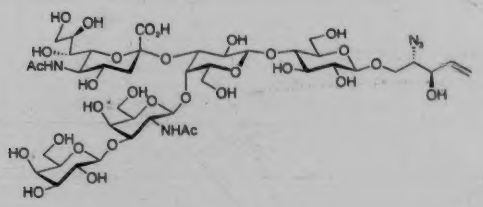
***O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-azido-pent-4-ene-1,3-diol (26).**

Compound **26** was prepared with bacterial strain ZWU12 using the general procedure for the production of oligosaccharide in high-cell density culture. Lactoside **11** (500 mg) was fed to the bacterial culture and incubated for 6 h. After isolation and purification of the oligosaccharide following the general procedure described above, the GM_2 derivative was obtained (316 mg): $[\alpha]_D$ +13.4° (*c* 0.41, H_2O). Selected ^1H NMR (600 MHz, D_2O): δ_H 5.92 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 6.7 Hz, H-4_{sphing}), 5.38 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 0.8 Hz, H-$5t_{sphing}$), 5.34 (ddd, 1H, $J_{5c,4}$ = 10.9 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 0.8 Hz, H-$5c_{sphing}$), 4.71 (d, 1H, overlap with D_2O, H-1_{GalNAc}), 4.54 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1_{Gal}), 4.52 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1_{Glc}), 4.36-4.34 (m, 1H, H-3_{sphing}), 4.15 (dd, 1H, $J_{3,2}$ = 9.7 Hz, $J_{3,4}$ = 3.0 Hz, H-3_{Gal}), 4.11 (d, 1H, $J_{4,3}$ = 3.0 Hz, H-4_{Gal}), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.4 Hz, $J_{6a,5}$ = 1.9 Hz, H-$6a_{Glc}$), 3.93-3.58 (m, H-4_{GalNAc}, H-2_{GalNAc}, H-$1a_{sphing}$, H-$1b_{sphing}$, H-2_{sphing}, H-$6b_{Glc}$, H-5_{GalNAc}, H-3_{GalNAc}, H-5_{Gal}, H-$9b_{Neu2}$, H-4_{Neu}, H-5_{Neu}, H-6_{Neu}, H-3_{Glc}, H-4_{Glc}, H-5_{Glc}), 3.48 (dd, 1H, $J_{7,8}$ = 10.1 Hz, $J_{7,6}$ = 2.1 Hz, H-7_{Neu}), 3.37-3.33 (m, 2H, H-2_{Gal}, H-2_{Glc}), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.6 Hz, $J_{3e,4}$ = 4.5 Hz, H-$3e_{Neu}$), 2.03, 2.01 (2 × s, 2 × 3H, 2 × $C(O)\underline{CH_3}$), 1.92 (dd, 1H, $J_{3a,3e} \approx J_{3a,4} \approx$ 11.9 Hz, H-$3a_{Neu}$); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 175.7, 174.9, 136.0, 119.6, 103.6, 103.5, 102.9, 102.5, 79.4, 78.0, 75.7, 75.6, 75.2, 74.9, 73.9, 73.5, 73.1, 72.2, 70.9, 69.6, 69.5, 68.9, 68.7, 65.8, 63.7, 62.0, 61.4, 60.9, 53.2, 52.5, 37.9, 23.5, 22.9. ESI HRMS calc'd for $C_{36}H_{58}N_5O_{25}$: 960.5326. Found: 960.3422.



O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→8)-O-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-O-β-D-galactopyranosyl-(1→4)]-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-azido-pent-4-ene-1,3-diol (27).

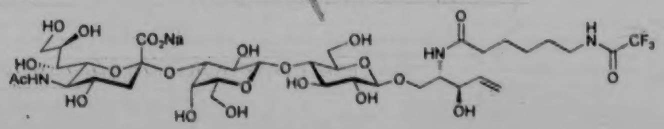
Compound **27** was prepared with bacterial strain ZWU12 using the general procedure for the production of oligosaccharide in high-cell density culture. Lactoside **11** (500 mg) was fed to the bacterial culture and incubated for 4 h. After isolation and purification of the oligosaccharide following the general procedure described above, the GD_2 derivative was obtained (207 mg): $[\alpha]_D$ +3.89° (*c* 0.53, H_2O). Selected ^{1}H NMR (600 MHz, D_2O): δ_H 5.93 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 6.8 Hz, H-4$_{\text{sphing}}$), 5.39 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 1.1 Hz, H-5t$_{\text{sphing}}$), 5.34 (ddd, 1H, $J_{5c,4}$ = 10.6 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 1.0 Hz, H-5c$_{\text{sphing}}$), 4.71 (d, 1H, $J_{1,2}$ = 8.4 Hz, H-1$_{\text{GalNAc}}$), 4.52 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{\text{Glc}}$), 4.51 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{\text{Gal}}$), 4.36-4.35 (m, 1H, H-3$_{\text{sphing}}$), 4.18-4.15 (m, 2H, H-3$_{\text{Gal}}$, H-9a$_{\text{Neu2}}$), 4.11-4.10 (m, 1H, H-8$_{\text{Neu2}}$), 4.04 (d, 1H, $J_{4,3}$ = 8.1 Hz, H-4$_{\text{Gal}}$), 4.00 (dd, 1H, $J_{6a,6b}$ = 10.2 Hz, $J_{6a,5}$ = 1.7 Hz, H-6a$_{\text{Glc}}$), 3.92-3.57 (m, H-4$_{\text{GalNAc}}$, H-2$_{\text{GalNAc}}$, H-2$_{\text{sphing}}$, H-1a$_{\text{sphing}}$, H-1b$_{\text{sphing}}$, H-6b$_{\text{Glc}}$, H-5$_{\text{GalNAc}}$, H-3$_{\text{GalNAc}}$, H-5$_{\text{Gal}}$, H-9b$_{\text{Neu2}}$, H-4$_{\text{NeuA}}$, H-4$_{\text{NeuB}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$, H-5$_{\text{Glc}}$), 3.40 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{2,1}$ = 8.0 Hz, H-2$_{\text{Gal}}$), 3.36-3.33 (m, 1H, H-2$_{\text{Glc}}$), 2.76 (dd, 1H, $J_{3e,3a}$ = 12.4 Hz, $J_{3e,4}$ = 4.7 Hz, H-3e$_{\text{NeuA}}$), 2.69 (dd, 1H, $J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 4.4 Hz, H-3e$_{\text{NeuB}}$), 2.08, 2.05, 2.03 (3 × s, 3 × 3H, 3 × C(O)$\underline{CH_3}$), 1.78 (dd, 1H, $J_{3a,3e} \approx J_{3a,4} \approx$ 12.3 Hz, H-3a$_{\text{NeuA}}$), 1.74 (dd, 1H, $J_{3a,3e} \approx J_{3a,4} \approx$ 12.3 Hz, H-3a$_{\text{NeuB}}$); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 175.83, 175.75, 174.3, 174.2, 136.0, 119.6, 103.6, 102.9, 101.5, 79.2, 75.8, 75.4, 75.2, 75.1, 73.6, 73.1, 72.6, 69.5, 69.4, 69.0, 65.8, 63.5, 62.4, 61.8, 61.5, 60.9, 53.4, 53.3, 52.7, 41.4, 40.1, 23.5, 23.3, 22.9. ESI HRMS calc'd for $C_{47}H_{74}N_6O_{33}$: 1205.4308. Found: 625.2155.



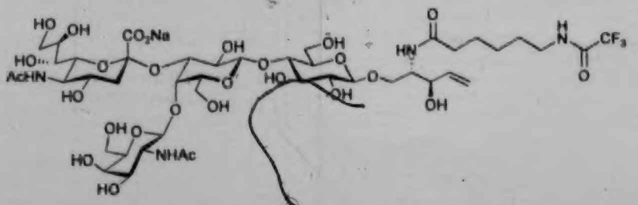
O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[O-(β-D-galactopyranosyl)-(1→3)-O-2-acetamido-2-deoxy-O-β-D-galactopyranosyl-(1→4)]-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-azido-pent-4-ene-1,3-diol (28).

Compound **28** was prepared with bacterial strain DC23 using the general procedure for the production of oligosaccharide in high-cell density culture. Lactoside **11** (500 mg) was fed to the bacterial culture and incubated for 23 h. After isolation and purification of the oligosaccharide following the general procedure described above, GM_1 derivative was obtained (356 mg): $[\alpha]_D$ +0.02° (*c* 0.53, H_2O). Selected ^1H NMR (600 MHz, D_2O): δ_H 5.92 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 6.8 Hz, H-4_{sphing}), 5.38 (ddd, 1H, $J_{5t,4}$ = 17.3 Hz, $J_{5t,5c} \approx J_{5t,3} \approx$ 1.1 Hz, H-$5t_{sphing}$), 5.34 (ddd, 1H, $J_{5c,4}$ = 10.5 Hz, $J_{5c,5t} \approx J_{5c,3} \approx$ 1.0 Hz, H-$5c_{sphing}$), 4.78 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1_{GalNAc}), 4.54 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Gal1}), 4.53 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal2}), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.36-4.34 (m, 1H, H-3_{sphing}), 4.16 (d, 1H, $J_{4,3}$ = 3.2 Hz, H-4_{Gal2}), 4.14-4.12 (m, 2H, H-4_{GalNAc}, H-3_{Gal2}), 4.04 (dd, 1H, $J_{2,3}$ = 10.9 Hz, $J_{2,1}$ = 8.6 Hz, H-2_{GalNAc}), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.0 Hz, H-$6a_{Glc}$), 3.91 (d, 1H, $J_{4,3}$ = 3.2 Hz, H-4_{Gal}), 3.88-3.86 (m, 3H, H-2_{sphing}, H-$1a_{sphing}$, H-$1b_{sphing}$), 3.85-3.71 (m, H-3_{GalNAc}, H-$6b_{Glc}$, H-4_{Neu}, H-5_{Gal2}, H-5_{GalNAc}), 3.66-3.58 (m, H-3_{Glc}, H-3_{Gal1}, H-4_{Glc}, H-5_{Glc},), 3.53-3.48 (m, 1H, H-2_{Gal1}), 3.37-3.33 (m, 2H, H-2_{Gal2}, H-2_{Glc}), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.6 Hz, $J_{3e,4}$ = 4.5 Hz, H-$3e_{Neu}$), 2.03, 2.00 (2 × s, 2 × 3H, 2 × -C(O)$\underline{CH_3}$), 1.93 (dd, 1H, $J_{3a,3e} \approx J_{3a,4} \approx$ 11.9 Hz, H-$3a_{Neu}$); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 175.6, 175.0, 136.0, 119.6, 105.6, 103.5, 103.4, 102.9, 102.5, 81.2, 79.4, 78.0, 75.8, 75.7, 75.2, 75.0, 74.0, 73.5, 73.4, 73.13, 73.10, 71.6, 70.9, 69.6, 69.54, 69.48, 68.9, 68.8, 65.8, 63.7, 62.0, 61.82, 61.79, 61.5, 61.0, 52.5, 52.1, 37.9, 23.5, 22.9. ESI HRMS calc'd for $C_{42}H_{68}N_5O_{30}$: 1122.3955. Found: 1122.3949.

Procedure (B) for deprotection and acylation of sphingosine amine using anhydride. Oligosaccharide **2** or **3** (17-40 µmol) was dissolved in dry methanol (2.0 mL). Sodium methoxide (~2 eq) was added and the mixture was stirred for 3 days at room temperature until completion of the reaction. The pH of the reaction mixture was adjusted to 9.0 using 0.5 M HCl and the methanol was evaporated. The compound was purified by HPLC (Hamilton semi-preparative PRP-1 column) using a MeOH/H_2O gradient. A portion of the amine (15-18 µmol), 6-trifluoroacetamidohexanoic anhydride (~5.0 eq), and triethylamine (2-4 eq) were dissolved in dry degassed methanol (3.0 mL). The solution was stirred overnight at room temperature. The mixture was concentrated, redissolved in water, and applied to a C18 Sep-Pak cartridge. Gradient elution (MeOH-H_2O) afforded the target compound, which was further purified by HPLC (Hamilton semi-preparative PRP-1 column) using a MeOH-H_2O gradient.

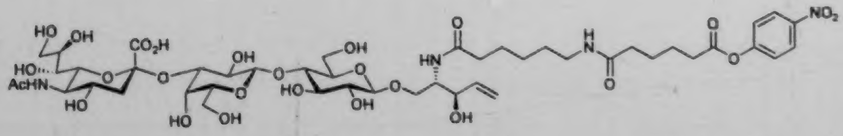


***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(6-trifluoroacetamidohexan-amido)-pent-4-ene-1,3-diol (30).** Compound **30** was prepared according to Procedure (B). Trisaccharide **2** (33.5 mg; 40.4 µmol) was deprotected and a portion of the amine (14.0 mg; 18.6 µmol) was acylated with 6-trifluoroacetamidohexanoic anhydride (40.5 mg; 92.8 µmol; 5.0 eq). Work-up and purification as described in Procedure (B) afforded **30** (13.9 mg; 14.4 µmol; 78%): $[\alpha]_D$ +16.8° (c 0.23, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.82 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{\mathrm{sphing}}$), 5.30 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.1 Hz, $J_{5t,3}$ = 1.1 Hz, H-5t$_{\mathrm{sphing}}$), 5.24 (ddd, 1H, $J_{5c,4}$ = 10.5 Hz, $J_{5c,5t}$ = 1.1 Hz, $J_{5c,3}$ = 1.1 Hz, H-5c$_{\mathrm{sphing}}$), 4.50 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{\mathrm{Gal}}$), 4.45 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{\mathrm{Glc}}$), 4.20 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3$_{\mathrm{sphing}}$), 4.10-4.03 (m, 3H, H-2$_{\mathrm{sphing}}$, H-3$_{\mathrm{Gal}}$, H-1a$_{\mathrm{sphing}}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 2.2 Hz, H-6a$_{\mathrm{Glc}}$), 3.94 (d, 1H, $J_{4,3}$ = 3.0 Hz, H-4$_{\mathrm{Gal}}$), 3.89-3.61 (m, 13H, H-8$_{\mathrm{Neu}}$, H-5$_{\mathrm{Neu}}$, H-9a$_{\mathrm{Neu}}$, H-6b$_{\mathrm{Glc}}$, H-1b$_{\mathrm{sphing}}$, H-4$_{\mathrm{Neu}}$, H-6a$_{\mathrm{Gal}}$, H-6b$_{\mathrm{Gal}}$, H-5$_{\mathrm{Gal}}$, H-3$_{\mathrm{Glc}}$, H-4$_{\mathrm{Glc}}$, H-9b$_{\mathrm{Neu}}$, H-6$_{\mathrm{Neu}}$), 3.59-3.54 (m, 3H, H-5$_{\mathrm{Glc}}$, H-7$_{\mathrm{Neu}}$, H-2$_{\mathrm{Gal}}$), 3.32-3.29 (m, 3H, H-2_{Glc}, $\underline{CH_2}$NHTFA), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.6 Hz, H-$3e_{Neu}$), 2.25 (t, 2H, J = 7.4 Hz, $\underline{CH_2}$C(O)), 2.02 (s, 3H, C(O)$\underline{CH_3}$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-$3a_{Neu}$), 1.62-1.55 (m, 4H, $\underline{CH_2}CH_2$NHTFA, $\underline{CH_2}CH_2$C(O)), 1.35-1.30 (m, 2H, $\underline{CH_2}$); ^{13}C NMR (125 MHz, D_2O) : δ_C 177.8, 175.9, 174.7, 159.7 (q, $J_{C,F}$ = 37 Hz), 137.2, 119.0, 116.8 (q, $J_{C,F}$ = 285 Hz), 103.6, 103.3, 100.7, 79.3, 76.4, 76.1, 75.7, 75.1, 73.8, 73.7, 72.8, 72.7, 70.3, 69.5, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 60.5, 53.8, 52.6, 40.5, 36.6, 28.3, 26.3, 25.8, 22.9. ESI HRMS calc'd for $C_{36}H_{57}N_3O_{22}F_3$: 940.3391. Found: 940.3385.

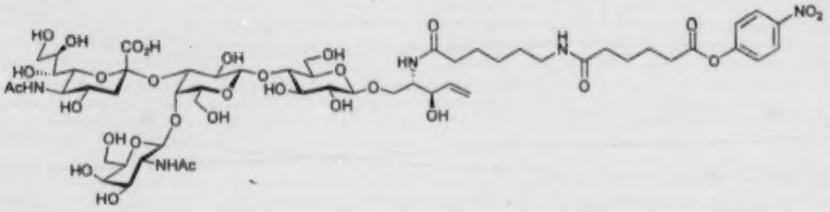


O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-O-β-D-galactopyranosyl-(1→4)]-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-(-6-trifluoroacetamidohexan-amido)-pent-4-ene-1,3-diol (31). Compound **31** was prepared according to Procedure (B). Tetrasaccharide **3** (18.8 mg; 17.8 μmol) was deprotected and a portion of the amine (14.7 mg, 15.3 μmol) was acylated with 6-trifluoroacetamidohexanoic anhydride (30.4 mg; 69.7 μmol; 5.0 eq). Work-up and purification as described in Procedure (B) afforded **31** (13.9 mg; 11.9 μmol; 78%): $[\alpha]_D$ +26.4° (c 0.14, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.82 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.28 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.0 Hz, $J_{5t,3}$ = 1.0 Hz, H-$5t_{sphing}$), 5.27 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.0 Hz, $J_{5c,3}$ = 1.0 Hz, H-$5c_{sphing}$), 4.72 (d, overlapped with HOD, 1H, H-1_{GalNAc}), 4.50 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 4.45 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.20 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3_{sphing}), 4.13 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 3.1 Hz, H-3_{Gal}), 4.10-4.04 (m, 3H, H-4_{Gal}, H-2_{sphing}, H-$1a_{sphing}$), 3.97 (dd, 1H, $J_{6a,6b}$ = 12.1 Hz, $J_{6a,5}$ = 1.6 Hz, H-$6a_{Glc}$), 3.92-3.88 (m, 2H, H-4_{GalNAc}, H-2_{GalNAc}) 3.85 (dd, 1H, $J_{9a,9b}$ = 12.0 Hz, $J_{9a,8}$ = 2.2 Hz, H-$9a_{Neu}$), 3.82-3.57 (m, 17H, H-5_{Neu}, H-$6b_{Glc}$, H-$1b_{sphing}$, H-4_{Neu}, H-5_{Gal}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{GalNAc}, H-$6a_{GalNAc}$, H-$6b_{GalNAc}$, H-8_{Neu}, H-3_{GalNAc}, H-3_{Glc}, H-$9b_{Neu}$, H-4_{Glc}, H-7_{Neu}, H-5_{Glc}), 3.47 (dd, 1H, $J_{6,5}$ = 10.1 Hz, $J_{6,7}$ = 2.1 Hz, H-6_{Neu}), 3.36-3.29 (m, 4H, H-2_{Gal}, H-2_{Glc}, $\underline{CH_2}$NHTFA), 2.65

(dd, 1H, $J_{3e,3a}$ = 12.1 Hz, $J_{3e,4}$ = 4.3 Hz, H-3e$_{Neu}$), 2.25 (t, 2H, J = 7.3 Hz, $\underline{CH_2}C(O)$), 2.02, 2.01 (2 × s, 2 × 3H, 2 × $C(O)\underline{CH_3}$), 1.91 (dd, 1H, $J_{3a,3e}$ = 12.1 Hz, $J_{3a,4}$ = 12.1 Hz, H-3a$_{Neu}$) 1.63-1.55 (m, 4H, $\underline{CH_2}CH_2NHTFA$, $\underline{CH_2}CH_2C(O)$), 1.35-1.29 (m, 2H, $\underline{CH_2}$); ^{13}C NMR (125 MHz, D_2O) : δ_C 177.8, 175.9, 175.7, 174.9, 159.7 (q, $J_{C,F}$ = 37 Hz), 137.2, 118.7, 116.8 (q, $J_{C,F}$ = 286 Hz), 103.6, 103.5, 103.3, 102.5, 79.6, 78.0, 75.6 (2 C), 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.2, 70.9, 69.6, 69.5, 69.0, 68.7, 63.7, 62.0, 61.4, 61.0, 53.8, 53.2, 52.5, 40.5, 37.9, 36.5, 28.3, 26.3, 25.8, 23.5, 22.9. ESI HRMS calc'd for $C_{44}H_{70}N_4O_{27}F_3Na_2$: 1189.3975. Found: 1189.3972.



O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-[6-(5-*p*-nitrophenyloxycarbonyl-pentan-amido)hexan-amido]-pent-4-ene-1,3-diol (32). Compound **30** was treated with a solution of sodium methoxide. The resulting amine (4.5 mg; 5.2 μmol) was dissolved in dimethylformamide (1.5 mL) and triethylamine (2.2 μL; 15.6 μmol; 3.0 eq) was added. *p*-Nitrophenyl adipate diester [245] (21.0 mg; 5.2 μmol; 10.0 eq) was added. After 15 min, the reaction was complete as observed by TLC (CH_2Cl_2-MeOH-H_2O-AcOH; 4:5:1:0.5). The pH was adjusted to 5.0 with acetic acid and the reaction mixture was co-evaporated with toluene (3 ×). The residue was suspended in 1% acetic acid solution and filtered through cotton. The filtrate was concentrated and the residue obtained was purified by HPLC (Beckman C_{18}-silica semi-preparative column) using a gradient of MeOH-H_2O containing 1% acetic acid. ^1H NMR spectrum was acquired in CD_3OD. Although visible, resonances were significantly broadened. ESI HRMS calc'd for $C_{46}H_{69}N_4O_{26}$: 1093.4195. Found: 1093.4189.



O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-O-β-D-galactopyranosyl-(1→4)]-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2S,3R)-2-[6-(5-*p*-nitrophenyloxycarbonyl-pentan-amido)hexan-amido]-pent-4-ene-1,3-diol (33). Compound **31** was treated with a solution of sodium methoxide. The resulting amine (3.4 mg; 3.2 μmol) was dissolved in dimethylformamide (1.0 mL) and triethylamine (2.0 μL; 14.2 μmol; 4.4 eq) was added. *p*-Nitrophenyl adipate diester[245] (12.5 mg; 32.2 μmol; 10 eq) was added. After 15 min, the reaction was complete as observed by TLC (CH_2Cl_2-MeOH-H_2O-AcOH; 4:5:1:0.5). The pH was adjusted to 5.0 with acetic acid and the reaction mixture was co-evaporated with toluene (3 ×). The residue was suspended in 1% acetic acid solution and filtered through cotton. The filtrate was concentrated and the residue obtained was purified by HPLC (Beckman C_{18}-silica semi-preparative column) using a gradient of MeOH-H_2O containing 1% acetic acid. ^{1}H NMR spectrum was recorded in CD_3OD. Although visible, the resonnances were significantly broadened. ESI HRMS calc'd for $C_{54}H_{82}N_5O_{31}Na_2$: 1342.4789. Found: 1342.4792.

Preparation of tetanus toxoid conjugates 34 and 35 with adipate linker.

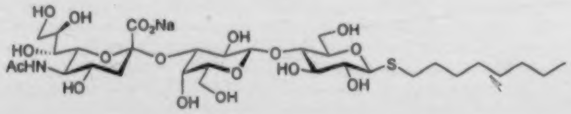
Purified monomeric tetanus toxoid (~6.6 mg/mL) in PBS, pH 7.2 (840 μL for **34**, 1.07 mL for **35**) was added to the activated sugars **32** or **33** (~30 eq). The mixture was gently tumbled overnight before being dialyzed against PBS (5 × 2 L) at 4°C. The incorporation level was determined by MALDI mass spectrometry. Results are presented in Scheme 4.1.

Preparation of bovine serum albumin conjugates 36 with adipate linker.

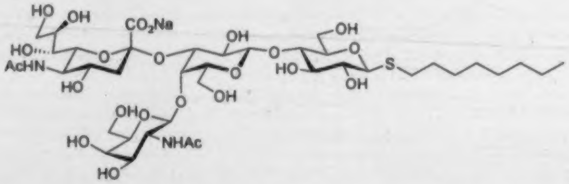
The activated sugar **32** (~30 eq) was dissolved in water and bovine serum albumin (4.3 mg) was added. The mixture was gently tumbled overnight before being dialyzed against water (5 × 2 L) at 4° C. The incorporation level was determined by MALDI mass spectrometry. Results are presented in Scheme 4.1.

Preparation of bovine serum albumin conjugates 40, 41, 42, 43, and 44 using diethyl squarate

Sugar amines **30**, **31**, **37**, **38**, and **39** were dissolved in methanol and 3,4-diethoxy-3-cyclobutene-1,2-dione (0.9 eq) was added in methanol. Reaction progress was monitored by TLC, and when necessary, sodium methoxide was added. When all the starting material had been consumed as judged by TLC, the mixture was transferred to an Ependorph tube and the solvent was remove with a gentle stream of argon. The residue was dried *in vacuo* for one hour, after which time a solution of BSA (5 mg/mL) in borate buffer, pH 9.0 was added. The solutions were tumbled overnight and the conjugates dialysed against milliQ water (5 × 2 L) at 4 °C.

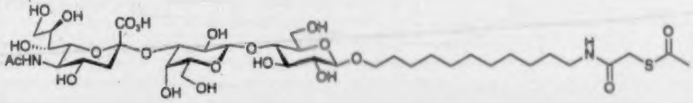


Octyl ***O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galacto-pyranosyl)-(1→4)-*O*-1-thio-β-D-glucopyranoside (46)**: The acceptor **45** (28.9 mg; 61.4 μmol; 12.3 mM in reaction), crude CMP-Neu5Ac (~89.3 μmol; ~17.9 mM in reaction; ~1.2 eq), $MnCl_2$ (100 μL of 0.5 M solution; 10 mM in reaction), $MgCl_2$ (100 μL of 0.5 M solution; 10 mM in reaction), dithiothreitol (37.5 μL of 0.1 M solution; 2.5 mM in reaction), 0.05 M HEPES buffer, pH 7.5 containing 10% (v/v) glycerol (2.25 mL), α-(2,3)-sialyltransferase (2.0 mL in 0.05 M HEPES buffer, pH 7.5 containing 10% (v/v) glycerol; ~1.25 U), and alkaline phosphatase (Roche Bioscience) (12.5 μL; ~12.5 U) were combined in a 45 mL Falcon tube. The mixture was tumbled gently at room temperature for 24 hr after which time TLC ($CHCl_3$-MeOH-H_2O-AcOH; 10:7:1:2) indicated almost complete disappearance of the starting material **45**. The reaction mixture was centrifuged at 14,000 rpm for 30 min and the supernatant applied to a Sep-Pak (tC18; 10g). Gradient elution (MeOH-H_2O) afforded **46**. Further purification by HPLC (Beckman C_{18}-silica semi-preparative column) using a gradient of water and methanol yielded **46** (40.6 mg; 53.3 μmol; 87%): $[\alpha]_D$ –28.9° (*c* 0.51, H_2O). ^1H NMR (500 MHz, D_2O): δ_H 4.52 (d, 2H, $J_{1,2}$ = 9.1 Hz, H-1_{Gal}, H-1_{Glc}), 4.10 (dd, 1H, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 3.0 Hz, H-3_{Gal}), 3.96 (m, 2H, H-$6a_{Glc}$, H-4_{Gal}), 3.91-3.79 (m, 7H, H-8_{Neu}, H-$9a_{Neu}$, H-5_{Gal}, H-$9b_{Neu}$, H-6_{Neu}, H-7_{Neu}, H-2_{Gal}), 3.75-3.56 (m, 6H, H-4_{Neu}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{Neu}, H-3_{Glc}, H-4_{Glc}), 3.37-3.33 (m, 1H, H-2_{Glc}), 2.78-2.70 (m, 2H, $\underline{CH_2}$S, H-$3e_{Neu}$), 2.03 (s, 3H, C(O)$\underline{CH_3}$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.1 Hz, $J_{3a,4}$ = 12.1 Hz, H-$3a_{Neu}$), 1.63 (quintet, 2H, *J* = 7.4 Hz, $\underline{CH_2}CH_2S$), 1.40-1.37 (m, 2H, $\underline{CH_2}CH_2CH_2S$), 1.32-1.26 (m, 8H, alkane $\underline{CH_2}$), 0.86 (t, 3H, *J* = 6.9 Hz, CH_3); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 174.7, 103.5, 100.7, 86.1, 79.5, 79.0, 76.7, 76.4, 76.1, 73.8, 72.9, 72.6, 70.2, 69.2, 69.0, 68.4, 63.5, 61.9, 61.1, 52.6, 40.5, 32.0, 30.8, 30.2, 29.1, 29.0, 28.8, 22.9, 14.3. ESI HRMS calc'd for $C_{31}H_{54}NO_{18}S$: 760.3067. Found:760.3065.



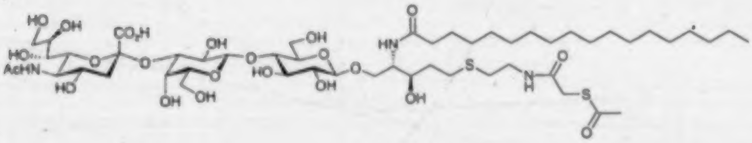
Octyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-1-thio-β-D-glucopyranoside (47) : The acceptor **46** (9.75 mg, 12.8 μmol) and UDP-*N*-acetylglucosamine disodium salt (11.7 mg, 18.0 μmol, 1.4 eq) were combined in a Falcon tube. UDP-*N*-acetylglucosamine 4-epimerase (1.0 mL, ~1.0 U) in 0.2 M NaCl, 1.0 mM EDTA, 20 mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 and *N*-acetylgalactosaminyl transferase (2.9 mL, ~1.0 U) were added to the mixture followed by 48 μL of 0.5 M $MgCl_2$ (6.0 mM in reaction) and alkaline phosphatase (10 μL, ~10 U). The reaction was gently tumbled for 16 hr until the reaction was completed as observed by silica gel TLC (EtOAc-MeOH-H_2O-AcOH; 6:3:3:2). The mixture was centrifuged for 20 min at 14,000 rpm, the supernatant was collected and applied to a Sep-Pak (tC18; 5g). Elution with a gradient of water-methanol afforded **47**. Further purification by HPLC (Beckman C_{18}-silica semi-preparative column) using a gradient of

water and methanol yielded **47** (10.8 mg, 11.3 μmol, 88%): $[\alpha]_D$ –5.71° (*c* 0.30, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 4.74 (m, 1H, H-1_{GalNAc}), 4.53-4.51 (m, 2H, H-1_{Gal}, H-1_{Glc}), 4.14-4.10 (m, 2H, H-3_{Gal}, H-4_{Gal}), 3.96-3.57 (m, 20H, H-$6a_{Glc}$, H-4_{GalNAc}, H-2_{GalNAc}, H-$9a_{Neu}$, H-5_{Neu}, H-$6b_{Glc}$, H-4_{Neu}, H-5_{Gal}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{GalNAc}, H-$6a_{GalNAc}$, H-$6b_{GalNAc}$, H-8_{Neu}, H-3_{GalNAc}, H-3_{Glc}, H-$9b_{Neu}$, H-4_{Glc}, H-7_{Neu}, H-5_{Glc}), 3.47 (dd, 1H, $J_{6,5}$ = 10.0 Hz, $J_{6,7}$ = 1.2 Hz H-6_{Neu}), 3.37-3.32 (m, 2H, H-2_{Gal}, H-2_{Glc}), 2.80-2.64 (m, 3H, $\underline{CH_2}S$, H-$3e_{Neu}$), 2.03, 2.01 (2 × s, 2 × 3H, 2 × $C(O)\underline{CH_3}$), 1.92 (dd, 1H, $J_{3a,3e}$ = 11.6 Hz, $J_{3a,4}$ = 11.6 Hz, H-$3a_{Neu}$), 1.64 (quintet, 2H, *J* = 7.3 Hz, $\underline{CH_2}CH_2S$), 1.40-1.37 (m, 2H, $\underline{CH_2}CH_2CH_2S$), 1.29-1.27 (m, 8H, alkane $\underline{CH_2}$), 0.86 (t, 3H, *J* = 6.7 Hz, CH_3); ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 175.7, 174.9, 103.6, 103.5, 102.5, 86.1, 80.0 (2C), 78.0, 76.7, 75.6, 75.2, 74.9, 4.0, 73.2, 72.9, 72.1, 70.9, 69.6, 69.0, 68.7, 62.1, 61.4, 61.2, 52.5, 51.8, 37.8, 32.0, 30.8, 30.2, 29.2, 29.0, 28.8, 23.5, 22.9, 14.3. ESI HRMS calc'd for $C_{39}H_{68}N_2O_{23}S$: 963.3861. Found: 963.3865.

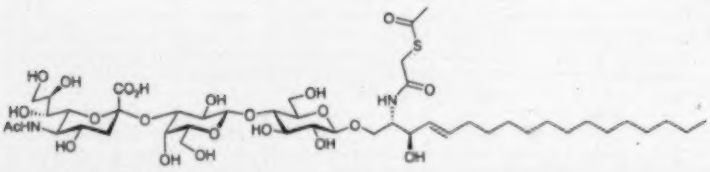


***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-11-*N*-(*S*-acetylthioacetate)-undecane (52)**. Sialoside **48** (7.5 mg; 9.3 μmol) and *N*-succinimidyl acetylthioacetate (6.48 mg; 28.0 μmol; 3.0 eq) were dissolved in a 9:1 pyridine-water mixture (1.0 mL). 4-*N*,*N*-Dimethylaminopyridine (3.42 mg; 28.0 μmol; 3.0 eq) was then added and the reaction mixture was stirred overnight at room temperature. After this time, the reaction mixture was concentrated to dryness. The residue obtained was resuspended in a solution of 10% MeOH/H_2O and applied to a C18 Sep-Pak cartridge. Elution with a gradient of MeOH/H_2O yielded the title compound **52** (6.2 mg; 6.7 μmol; 73%): Selected ^{1}H NMR (500 MHz, CD_3OD): δ_H 4.41 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Gal}), 4.25 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Glc}), 4.03 (dd, 1H, $J_{6a,6b}$ = 9.5 Hz, $J_{6a,5}$ = 3.3 Hz, H-$6a_{Glc}$), 3.89 (dd, 1H, $J_{6b,6a}$ = 9.7 Hz, $J_{6b,5}$ = 2.8 Hz, H-$6b_{Glc}$), 3.86-3.39 (m, H-4_{Gal}, H-8_{Neu}, H-$9a_{Neu}$, H-5_{Gal}, -$\underline{CH_2}SC(O)CH_3$, H-$9b_{Neu}$, H-$6_{Neu}$, H-$7_{Neu}$, H-$4_{Neu}$, H-$6a_{Gal}$, H-$6b_{Gal}$, H-$5_{Neu}$, H-$2_{Gal}$, H-$5_{Glc}$, H-$4_{Glc}$, H-$3_{Glc}$), 3.24-3.20 (m, 3H, H-$2_{Glc}$, -$CH_2NH$-), 2.85-

2.81 (m, 1H, H-3e$_{Neu}$), 2.14 (s, 3H, SC(O)C$\underline{H}_3$), 1.99 (s, 3H, NHC(O)C$\underline{H}_3$), 1.74-1.69 (m, 1H, H-3a$_{Neu}$), 1.60 (p, 2H, J = 6.6 Hz, -C$\underline{H}_2$CH$_2$O-), 1.52 (p, 2H, J = 4.8 Hz, -C$\underline{H}_2$CH$_2$NH-), 1.39-1.27 (m, 14H, -C$\underline{H}_2$-)



***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-8-amino-2-*N*-octadecylamido-6-thia-octane-1,3-diol (53)**. Sialoside **49** (10.0 mg; 9.11 μmol) and *N*-succinimidyl acetylthioacetate (5.26 mg; 22.76 μmol; 2.5 eq) were dissolved in a 9:1 pyridine-water mixture (1.0 mL). 4-*N*,*N*-Dimethylaminopyridine (1.11 mg; 9.11 μmol; 1.0 eq) was then added and the reaction mixture was stirred overnight at room temperature. After this time, the solvent was evaporated *in vacuo*. The residue obtained was resuspended in a solution of 10% MeOH/H$_2$O and applied to a C18 Sep-Pak cartridge. Elution with a gradient of MeOH/H$_2$O afforded the target compound **53** (8.0 mg; 6.7 μmol; 74%): Selected ^{1}H NMR (500 MHz, CD$_3$OD): δ_H 4.40 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{Gal}$), 4.28 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{Glc}$), 4.17 (dd, 1H, $J_{1a,1b}$ = 10.2 Hz, $J_{1a,2}$ = 4.4 Hz, H-1a$_{sphing}$), 4.03 (dd, 1H, $J_{6a,6b}$ = 9.7 Hz, $J_{6a,5}$ = 3.1 Hz, H-6a$_{Glc}$), 3.94-3.50 (m, H-1b$_{sphing}$, H-6b$_{Glc}$, H-4$_{Neu}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{Gal}$, C(O)C$\underline{H}_2$SAc, H-2$_{Gal}$, H-3$_{Glc}$, H-5$_{Glc}$, H-4$_{Glc}$, H-9b$_{Neu}$), 3.34 (dd, 1H, $J_{2,1} \approx J_{2,3} \approx$ 7.1 Hz, H-2$_{Glc}$), 2.84 (m, 1H, H-3e$_{Neu}$), 2.72 (m, 1H, H-5a$_{sphing}$), 2.60 (dd, 1H, $J_{5a,5b} \approx J_{5a,4} \approx$ 6.0 Hz, H-5b$_{sphing}$), 2.35 (s, 3H, -SC(O)C$\underline{H}_3$), 2.21 (s, 3H, -HNC(O)C$\underline{H}_3$), 1.81-1.57 (m, 5H, H-4a$_{sphing}$, H-4b$_{sphing}$, H-3a$_{Neu}$, -C$\underline{H}_2$-), 1.32-1.27 (m, 28H, -C$\underline{H}_2$-), 0.89 (t, 3H, J = 7.0 Hz, -CH$_3$); ^{13}C NMR (125 MHz, CD$_3$OD): δ_C 176.3, 175.5, 170.6, 108.2, 105.2, 104.5, 101.1, 80.9, 77.7, 77.1, 76.5, 76.3, 75.0, 74.8, 73.0, 70.9, 70.20, 70.17, 70.0, 69.4, 69.0, 64.7, 62.8, 61.8, 54.8, 54.0, 42.1, 40.7, 37.3, 35.03, 35.01, 33.7, 33.1, 31.9, 30.8, 30.77, 30.70, 30.67, 30.53, 30.47, 33.1, 31.9, 30.80, 30.77, 30.70, 30.67, 30.53, 30.47, 30.41, 30.1, 29.0, 27.1, 23.7, 22.6, 14.4. HRMS calc'd for C$_{52}$H$_{92}$N$_3$O$_{23}$S$_2$: 1190.5569. Found: 1190.5561.



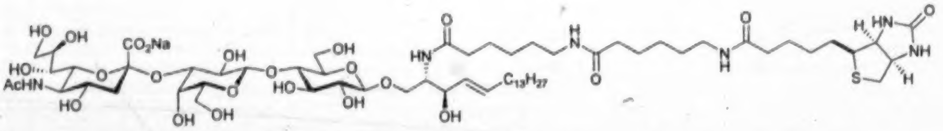
***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-*N*-(*S*-acetylthioacetate)-undecan-4-ene-1,3-diol (54)**. Glycolipid **50** (7.5 mg; 8.2 μmol) and *N*-succinimidyl acetylthioacetate (5.69 mg; 24.6 μmol; 3.0 eq) were dissolved in a 9:1 pyridine-water mixture (1.0 mL). 4-*N*,*N*-Dimethylaminopyridine (3.01 mg; 24.61 μmol; 3.0 eq) was then added and the reaction mixture was stirred overnight at room temperature. After this time, the reaction mixture was concentrated to dryness. The residue obtained was resuspended in a solution of 10% MeOH/H_2O and applied to a C18 Sep-Pak cartridge. Elution with a gradient of MeOH/H_2O yielded the title compound **54** (7.9 mg; 7.7 μmol; 94%): Selected ^{1}H NMR (500 MHz, CD_3OD): δ_H ^{1}H NMR (500 MHz, CD_3OD): δ_H 4.40 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Gal}), 4.28 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Glc}), 4.17 (dd, 1H, $J_{1a,1b}$ = 10.2 Hz, $J_{1a,2}$ = 4.4 Hz, H-$1a_{sphing}$), 4.03 (dd, 1H, $J_{6a,6b}$ = 9.7 Hz, $J_{6a,5}$ = 3.1 Hz, H-$6a_{Glc}$), 3.94-3.50 (m, H-$1b_{sphing}$, H-$6b_{Glc}$, H-4_{Neu}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{Gal}, C(O)C$\underline{H}_2$SAc, H-2_{Gal}, H-3_{Glc}, H-5_{Glc}, H-4_{Glc}, H-$9b_{Neu}$), 3.34 (dd, 1H, $J_{2,1} \approx J_{2,3} \approx$ 7.1 Hz, H-2_{Glc}), 2.84 (m, 1H, H-$3e_{Neu}$), 2.72 (m, 1H, H-$5a_{sphing}$), 2.60 (dd, 1H, $J_{5a,5b} \approx J_{5a,4} \approx$ 6.0 Hz, H-$5b_{sphing}$), 2.35 (s, 3H, -SC(O)C$\underline{H}_3$), 2.21 (s, 3H, -HNC(O)C$\underline{H}_3$), 1.81-1.57 (m, 5H, H-$4a_{sphing}$, H-$4b_{sphing}$, H-$3a_{Neu}$, -C$\underline{H}_2$-), 1.32-1.27 (m, 28H, -C$\underline{H}_2$-), 0.89 (t, 3H, J = 7.0 Hz, -CH_3); ^{13}C NMR (125 MHz, CD_3OD): δ_C 176.3, 175.5, 170.6, 108.2, 105.2, 104.5, 101.1, 80.9, 77.7, 77.1, 76.5, 76.3, 75.0, 74.8, 73.0, 70.9, 70.20, 70.17, 70.0, 69.4, 69.0, 64.7, 62.8, 61.8, 54.8, 54.0, 42.1, 40.7, 37.3, 35.03, 35.01, 33.7, 33.1, 31.9, 30.8, 30.77, 30.70, 30.67, 30.53, 30.47, 33.1, 31.9, 30.80, 30.77, 30.70, 30.67, 30.53, 30.47, 30.41, 30.1, 29.0, 27.1, 23.7, 22.6, 14.4. HRMS calc'd for $C_{52}H_{92}N_3O_{23}S_2$: 1190.5569. Found: 1190.5561.

Bromoacetylation of tetanus toxoid

Purified tetanus toxoid monomer (1.08 mL, ~9.76 mg/mL) in 20 mM PBS, pH 7.2) was treated with *N*-succimidyl bromoacetate (35 μL, 0.267 M). The solution was gently tumbled in the dark, at room temperature for 30 min. After this time, the protein solution was treated with a second portion of *N*-succimidyl bromoacetate (35 μL, 0.267 M) and again tumbled in the dark, at room temperature for 30 min. The derivatized protein was then purified by size exclusion chromatography (PD-10 desalting column) using sodium 20 mM phosphate buffer, pH 7.0 containing 1.0 M EDTA. The protein was used without any further processing in conjugation reactions.

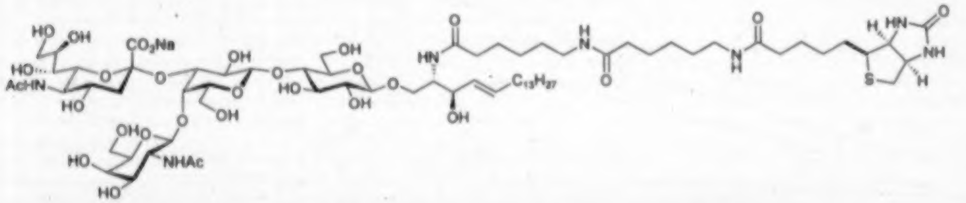
Preparation of tetanus toxoid conjugates 58, 59 and 60.

The oligosaccharide derivatives (3.5 mg for **52**, 2.6 mg for **53** and **54**) were first deprotected by incubation with a deacetylation solution (300 μL) composed of 0.5 M hydroxylamine, 25 mM EDTA in PBS, pH 7.2). After 3hrs, the sugar solution was added to the derivatized tetanus toxoid solution and gently mixed overnight. The glycoconjugates were then dialyzed against PBS (5 × 2 L) at 4°C. The incorporation levels were determined by MALDI mass spectrometry. Results are presented in Scheme 4.4.



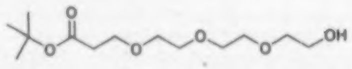
***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-*N*-(6-[[6-hexahydro-2-oxo-1-thieno [3,4-*d*] imidazol-4-yl-1-oxohexyl]amino])-undecan-4-ene-1,3-diol (64)**. Glycolipid **50** (10.0 mg; 10.7 μmol) and 1H-Thieno[3,4-d]imidazole-4-pentanamide (9.7 mg; 21.3 μmol; 2.0 eq) were dissolved in a 9:1 solution of pyridine and water. 4-*N*,*N*-Dimethylaminopyridine (1.3 mg; 10.6 μmol; 1.0 eq) was added and the reaction mixture was stirred overnight at room temperature. After completion of the reaction, the solvent was evaporated. The residue obtained was redissolved in milliQ water and applied to a C18 Sep-Pak cartridge. The targeted ganglioside was eluted with a gradient of methanol/water and lyophilized to obtain **64** (10.5 mg; 8.4 μmol; 78%):

Selected ^{1}H NMR (500 MHz, CD$_3$OD): δ_H 5.66 (ddd, 1H, $J_{5,4}$ = 15.0 Hz, $J_{5,6a} \approx J_{5,6b}$ 7.3 Hz, H-5$_{\text{Sphing}}$), 5.43 (ddd, 1H, $J_{4,5}$ = 14.9 Hz, $J_{4,3}$ = 7.4Hz, $J_{4,6}$ = 1.3 Hz, H-4$_{\text{Sphing}}$), 4.47 (dd, 1H, $J_{7,6}$ = 7.7 Hz, $J_{7,8a}$ = 5.1 Hz H-7$_{\text{Bio}}$), 4.41 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{\text{Gal}}$), 4.30-4.27 (m, 2H, H-1$_{\text{Glc}}$, H-6$_{\text{Bio}}$), 4.08-4.02 (m, 2H, H-3$_{\text{Sphing}}$, H-3$_{\text{Gal}}$), 4.02-3.95 (m, 1H, H-2$_{\text{Sphing}}$), 3.96-3.50 (m, H-4$_{\text{Gal}}$, H-6a$_{\text{Glc}}$, H-4$_{\text{Neu}}$, H-5$_{\text{Neu}}$, H-1a$_{\text{sphing}}$,H-2$_{\text{Gal}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$, H-5$_{\text{Glc}}$), 3.21-3.13 (m, 2H, H-5$_{\text{Bio}}$, H-2$_{\text{Glc}}$), 2.91 (dd, 1H, $J_{8a,8b}$=12.8 Hz, $J_{8a,7}$ = 5.0 Hz, H-8a$_{\text{Bio}}$), 2.84 (br d, 1H, $J_{3e,3a}$=11.6 Hz, H-3e$_{\text{Neu}}$), 2.69 (d, 1H, $J_{8b,8a}$=12.5 Hz, H-8b$_{\text{Bio}}$), 2.18 (t, 1H, $J_{1,2}$ = 7.2 Hz, H-1$_{\text{Bio}}$), 2.02-2.00 (m, 1H, H-6$_{\text{sphing}}$), 2.00 (s, 3H, C(O)C$\underline{H_3}$), 1.75-1.31 (m, 4H, H-3a$_{\text{Neu}}$, H-4a$_{\text{Bio}}$, H-4b$_{\text{Bio}}$, H-3$_{\text{Bio}}$, -CH$_2$-), 0.89 (t, 3H, J =7.0 Hz, -CH$_3$); ^{13}C NMR (125 MHz, D$_2$O): δ_C 175.94, 175.85, 175.5, 134.9, 131.3, 105.2, 104.6, 81.0, 77.7, 77.1, 76.5, 76.3, 75.0, 74.9, 73.1, 73.0, 70.9, 70.2, 69.8, 69.4, 69.0, 64.7, 63.4, 62.8, 61.9, 61.7, 57.0, 54.9, 54.0, 42.1, 41.1, 40.3, 40.1, 37.1, 36.9, 33.4, 33.1, 30.81, 30.77, 30.7, 30.5, 30.4, 30.3, 30.2, 29.8, 29.5, 27.7, 26.9, 26.7, 23.7, 22.6, 14.4. ESI HRMS calc'd for $C_{57}H_{98}N_5O_{28}S$: 1252.6368. Found 1252.6368.

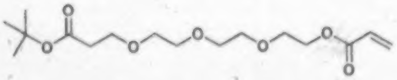


***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*)-2-*N*-(6-[[6-hexahydro-2-oxo-1-thieno [3,4-*d*] imidazol-4-yl-1-oxohexyl]amino])-undecan-4-ene-1,3-diol (65)**. Compound **67** (2.97 mg; 2.60 μmol) and 1H-Thieno[3,4-d]imidazole-4-pentanamide (2.37 mg; 5.21 μmol; 2.0 eq) were dissolved in a 9:1 solution of pyridine and water. 4-*N*,*N*-Dimethylaminopyridine (1.3 mg; 10.6 μmol; 4.1 eq) was added and the reaction mixture was stirred overnight at room temperature. After completion of the reaction, the solvent was evaporated. The residue obtained was redissolved in milliQ water and applied to a C18 Sep-Pak cartridge. The targeted ganglioside was eluted with a gradient of methanol/water and lyophilized to obtain **65** (2.0 mg; 2.0 μmol; 76%): Selected ^{1}H NMR (500 MHz, CD$_3$OD): δ_H 5.66 (ddd, 1H, $J_{5,4}$ = 15.0 Hz, $J_{5,6a} \approx J_{5,6b}$ 7.3

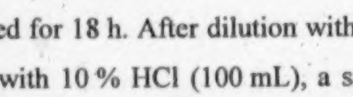
Hz, H-5$_{\text{Sphing}}$), 5.43 (ddd, 1H, $J_{4,5}$ = 14.9 Hz, $J_{4,3}$ = 7.4Hz, $J_{4,6}$ = 1.3 Hz, H-4$_{\text{Sphing}}$), 4.47 (dd, 1H, $J_{7,6}$ = 7.7 Hz, $J_{7,8a}$ = 5.1 Hz H-7$_{\text{Bio}}$), 4.41 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{\text{Gal}}$), 4.30-4.27 (m, 2H, H-1$_{\text{Glc}}$, H-6$_{\text{Bio}}$), 4.08-4.02 (m, 2H, H-3$_{\text{Sphing}}$, H-3$_{\text{Gal}}$), 4.02-3.95 (m, 1H, H-2$_{\text{Sphing}}$), 3.96-3.50 (m, H-4$_{\text{Gal}}$, H-6a$_{\text{Glc}}$, H-4$_{\text{Neu}}$, H-5$_{\text{Neu}}$, H-1a$_{\text{sphing}}$,H-2$_{\text{Gal}}$, H-3$_{\text{Glc}}$, H-4$_{\text{Glc}}$, H-5$_{\text{Glc}}$), 3.21-3.13 (m, 2H, H-5$_{\text{Bio}}$, H-2$_{\text{Glc}}$), 2.91 (dd, 1H, $J_{8a,8b}$=12.8 Hz, $J_{8a,7}$ = 5.0 Hz, H-8a$_{\text{Bio}}$), 2.84 (br d, 1H, $J_{3e,3a}$=11.6 Hz, H-3e$_{\text{Neu}}$), 2.69 (d, 1H, $J_{8b,8a}$=12.5 Hz, H-8b$_{\text{Bio}}$), 2.18 (t, 1H, $J_{1,2}$ = 7.2 Hz, H-1$_{\text{Bio}}$), 2.02-2.00 (m, 1H, H-6$_{\text{sphing}}$), 2.00 (s, 3H, C(O)C$\underline{H}_3$), 1.75-1.31 (m, H-3a$_{\text{Neu}}$, H-4a$_{\text{Bio}}$, H-4b$_{\text{Bio}}$, H-3$_{\text{Bio}}$, -CH$_2$-), 0.89 (t, 3H, J=7.0 Hz, -CH$_3$); ^{13}C NMR (125 MHz, D$_2$O): δ_C 175.94, 175.85, 175.5, 134.9, 131.3, 105.2, 104.6, 81.0, 77.7, 77.1, 76.5, 76.3, 75.0, 74.9, 73.1, 73.0, 70.9, 70.2, 69.8, 69.4, 69.0, 64.7, 63.4, 62.8, 61.9, 61.7, 57.0, 54.9, 54.0, 42.1, 41.1, 40.3, 40.1, 37.1, 36.9, 33.4, 33.1, 30.81, 30.77, 30.7, 30.5, 30.4, 30.3, 30.2, 29.8, 29.5, 27.7, 26.9, 26.7, 23.7, 22.6, 14.4. ESI HRMS calc'd for $C_{57}H_{98}N_5O_{28}S$: 1252.6368. Found 1252.6368.



12-Hydroxy-4,7,10-trioxadodecanoic acid-tert-butylester (68): Sodium metal (40 mg, 0.9 mmol) was added under argon to a solution of anhydrous triethylene glycol (25.6 mL, 188.0 mmol) in dry THF (85 mL). After complete dissolution of the metal, tert-butyl acrylate (9.6 mL, 66.0 mmol) was added, and the mixture was stirred at room temperature for 20 h. The solution was neutralized by the addition of 1 N HCl (1.6 mL) and subsequently concentrated *in vacuo*. The resulting residue was taken up in brine (70 mL), and extracted with ethyl acetate (3×50 mL). The combined organic phases were washed with brine, dried over magnesium sulfate, and concentrated *in vacuo*. The crude product **68** (16.4 g, 58.9 mmol, 89 %), which was obtained as a clear, colorless solution was used for the subsequent reaction step without further purification: R_f=0.30 (ethyl acetate); ^{1}H NMR (600 MHz, CDCl$_3$): δ_H 3.73-3.69 (m, 4 H, 3-CH2, 11-CH$_2$), 3.69-3.63 (m, 6 H, 3×OCH$_2$), 3.62 (m, 4 H, 12-CH$_2$, -OCH$_2$), 2.50 (t, 2 H, $J_{CH2,CH2}$ = 6.6 Hz, 2-CH$_2$), 1.44 (s, 9 H, CH$_3$-tBu); ^{13}C NMR (125 MHz, CDCl$_3$): δ_C 170.9, 80.5, 70.6, 70.51, 70.4, 70.4, 66.9, 61.8, 36.2, 28.17, 28.16, 28.09. ESI HRMS calc'd for $C_{13}H_{26}O_6Na$: 301.1622. Found: 301.1621.

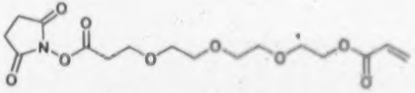


14-Oxo-4,7,10,13-tetraoxahexadec-15-enoic acid-tert-butylester (69): Under ice cooling, acryloyl chloride (3.40 g, 37.60 mmol) was added dropwise to a solution of **68** (6.00 g, 21.6 mmol) and *N,N*-diisopropylethylamine (4.68 g, 36.2 mmol) in dry dichloromethane (100 mL) at 0 °C. The solution was allowed to warm up to room temperature over 1 h, and stirred for 18 h. After dilution with ethyl acetate (100 mL), the reaction mixture was washed with 10 % HCl (100 mL), a saturated solution of sodium bicarbonate (100 mL) and brine (100 mL). The organic phase was dried over magnesium sulfate and concentrated under vaccum. The crude product was purified by silica gel column chromatography (hexanes/ethyl acetate 3:1) to give compound **69** (5.85 g, 17.6 mmol, 82 %) as a slightly yellow oil: ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 6.42 (dd, 1 H, $J_{2t,1}$ = 17.4 Hz, $J_{2t,2c}$ = 1.2 Hz, H-2t$_{acrylate}$), 6.15 (dd, 1 H, $J_{1,2t}$ = 17.4 Hz, $J_{1,2c}$ = 10.7 Hz, H-1$_{acrylate}$), 5.82 (dd, 1 H, $J_{2c,1}$ = 10.4 Hz, $J_{2c,2t}$ = 1.4 Hz, H-2c$_{acrylate}$), 4.32-4.29 (m, 2 H, 12-CH_2), 3.75-3.58 (m, 12 H, 6 × CH_2), 2.49 (t, 2 H, $J_{CH2,CH2}$ = 6.4 Hz, 2-CH_2), 1.44 (s, 9 H, CH_3-tBu); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 170.9, 166.1, 130.9, 128.3, 80.5, 70.64, 70.63, 70.57, 70.4, 69.1, 66.9, 63.6, 36.3, 28.1. ESI HRMS calc'd for $C_{16}H_{28}O_7Na$: 355.1727. Found: 355.1725.

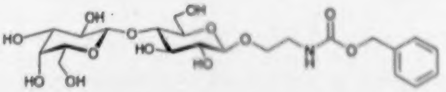


14-Oxo-4,7,10,13-tetraoxahexadec-15-enoic acid (70): A solution of protected linker **69** (2.50 g, 7.52 mmol) in a mixture of dichloromethane (5 mL), TFA (4.5 mL) and anisole (0.5 mL) was stirred at room temperature for 2 h. The reaction mixture was then diluted with toluene (25 mL), and the solvent was removed *in vacuo*. The resulting residue was co-evaporated with toluene (3 × 25 mL) and purified by column chromatography (hexanes/ethyl acetate 3:1 → 1:1 ethyl acetate) to yield compound **70** as a colorless oil (1.70 g, 6.15 mmol, 82 %): R_f = 0.15 (hexanes/ethyl acetate 1:1). ^{1}H NMR (600 MHz, CD_3OD): δ_H 6.39 (dd, 1 H, $J_{2t,1}$ = 17.4 Hz, $J_{2t,2c}$ = 1.2 Hz, H-2t$_{acrylate}$), 6.17 (dd, 1 H, $J_{1,2t}$ = 17.4 Hz, $J_{1,2c}$ = 10.2 Hz, H-1$_{acrylate}$), 5.88 (dd, 1 H, $J_{2c,1}$ = 10.2 Hz, $J_{2c/2t}$ = 1.8 Hz, H-

2c$_{acrylate}$), 4.30-4.26 (m, 2 H, 12-CH$_2$), 3.73-3.69 (m, 4 H, 3-CH$_2$, 11-CH$_2$), 3.66-3.56 (m, 8 H, 3 × OCH$_2$, 12-CH$_2$), 2.53 (t, 2 H, $J_{CH2,CH2}$ = 6.0 Hz, 2-CH$_2$); ^{13}C NMR (125 MHz, CD$_3$OD): δ_C 175.3, 167.6, 131.7, 129.5, 71.6, 71.5, 71.4, 67.9, 35.8. ESI HRMS calc'd for $C_{12}H_{20}O_7Na$: 299.1101. Found: 299.1098.

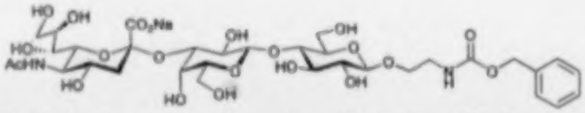


14-Oxo-4,7,10,13-tetraoxahexadec-15-enoic acid-N-hydroxysuccinimide ester (71): *N*,*N'*-Dicyclohexylcarbodiimide (248 mg, 1.20 mmol) and *N*-hydroxysuccinimide (142 mg, 1.20 mmol) were added under argon to a solution of **70** (300 mg, 1.09 mmol) in dry THF (9 mL). The reaction mixture was stirred at room temperature for 15 h. After concentration under reduced pressure, the resulting residue was purified by column chromatography to afford **71** as a colorless oil (292 mg, 0.78 mmol, 72 %): R$_f$ = 0.40 (hexanes/ethyl acetate 1:1); ^{1}H NMR (600 MHz, CDCl3): δ_H 6.43 (dd, 1 H, , $J_{2t,1}$ = 17.4 Hz, $J_{2t,2c}$ = 1.2 Hz, H-2t$_{acrylate}$), 6.16 (dd, 1 H, , $J_{1,2t}$ = 17.4 Hz, $J_{1,2c}$ = 10.8 Hz, H-1$_{acrylate}$), 5.84 (dd, 1 H, $J_{2c,1}$ = 10.5 Hz, $J_{2c,2t}$ = 1.2 Hz, H-2c$_{acrylate}$), 4.34-4.29 (m, 2 H, 12-CH$_2$), 3.85 (t, 2 H, $J_{CH2,CH2}$ = 6.6 Hz, CH$_2$), 3.75-3.72 (m, 2 H, CH$_2$), 3.69-3.61 (m, 8 H, 4 × CH$_2$), 2.90 (t, 2 H, $J_{2,3}$ = $J_{2,2'}$ = 6.0 Hz, 2-CH$_2$), 2.83 ppm (br s, 4 H, CH$_2$-NHS); ^{13}C NMR (125 MHz, CDCl3): δ_C 168.9, 166.7, 166.1, 130.9, 128.3, 70.7, 70.6, 70.60, 70.55, 69.1, 32.2, 25.6, 25.6. ESI HRMS calc'd for $C_{16}H_{23}NO_9Na$: 396.1265. Found: 396.1262.

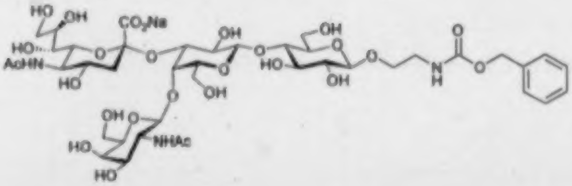


***O*-(β-D-Galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-2-*N*-(benzyloxycarbonyl)-aminoethanol (72)**. Perbenzoylated lactoside[290] (1.18 g; 943 μmol) was suspended in dry methanol (20 mL) and a catalytic amount of sodium metal was added. The reaction mixture was stirred at room temperature for 12 hr. After this time, the solution was neutralized to pH 7 using an H$^+$ ion-exchange resin (Rexyn 101). The resin was then filtered off and the filtrate was concentrated. The residue was dissolved in water, separated in two portions, and applied to a Sep-Pak (C18; 10 g). Gradient elution (MeOH-H$_2$O) afforded **72** (386 mg; 743 μmol; 80%): [α]$_D$ +1.1° (*c* 0.27,

H_2O). ^1H NMR (500 MHz, D_2O): δ_H 7.44-7.41 (m, 5H, Ar-$\underline{H}$), 5.11 (br s, 2H, ArC$\underline{H}_2$O-), 4.43 (d, 1H, $J_{1,2}$ = 8.4 Hz, H-1_{Glc}), 4.41 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 3.98-3.88 (m, 3H, H-4_{Gal}, H-$6b_{Glc}$, -C$\underline{H}_2$O-), 3.80-3.69 (m, 5H, -C$\underline{H}_2$O- H-$6b_{Glc}$, H-5_{Gal}, H-$6a_{Gal}$, H-$6b_{Gal}$), 3.64 (dd, 1H, $J_{3,2}$ = 10.0 Hz, $J_{3,4}$ = 3.4 Hz, H-3_{Gal}), 3.62-3.59 (m, 1H, H-3_{Glc}), 3.55-3.51 (m, 2H, H-2_{Gal}, H-4_{Glc}), 3.37-3.33 (m, 2H, -CH_2NH-), 3.31-3.27 (m, 1H, H-2_{Glc}); ^{13}C NMR (125 MHz, D_2O): δ_C 159.3, 137.4, 129.7, 129.3, 128.7, 103.8, 103.2, 79.3, 76.2, 75.6, 75.2, 73.7, 73.4, 71.8, 69.9, 69.4, 67.8, 61.9, 61.0, 41.4. ESI HRMS calc'd for $C_{22}H_{33}NO_{13}Na$: 542.1844. Found: 542.1845.

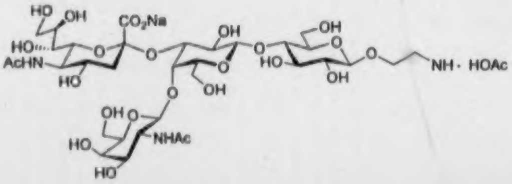


***O*-(5-Acetamino-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-2-*N*-(benzyloxycarbonyl)-aminoethanol (73)**. The acceptor **72** (49.2 mg; 94.7 μmol; 9.47 mM in reaction), crude CMP-Neu5Ac (80.6 mg; 122 μmol; 12.2 mM in reaction; 1.3 eq), $MnCl_2$ (200 μL of a 0.5 M solution; 10 mM in reaction), $MgCl_2$ (200 μL of a 0.5 M solution; 10 mM in reaction), dithiothreitol (250 μL of a 0.1 M solution; 2.5 mM in reaction), water (1.2 mL), α-(2,3)-sialyltransferase (9.0 mL; ~ 1.35 U), and alkaline phosphatase (Roche Bioscience) (9.0 μL; ~ 9.0 U) in 0.05 M HEPES buffer, pH 7.5 containing 10 % (v/v) glycerol were combined in a 15 mL Falcon tube. The mixture was tumbled gently at room temperature for 24 hr. after which time TLC (CH_2Cl_2-MeOH-H_2O-AcOH; 5:3:1:1) indicated almost complete disappearance of the starting material **72**. The reaction mixture was centrifuged at 14 000 rpm for 5 min. and the supernatant applied to a Sep-Pak (C18; 10 g). Gradient elution (MeOH-H_2O) afforded **73**. Further purification by size exclusion chromatography (Biogel P-4 resin; 75 cm × 2 cm) using H_2O as an eluent yielded **73** (67.9 mg; 81.5 μmol; 86%): $[\alpha]_D$ – 0.3° (*c* 0.20, H_2O). ^1H NMR (600 MHz, D_2O): δ_H 7.45-7.40 (m, 5H, Ar-H), 5.12 (br s, 2H, ArCH_2O-), 4.49 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 4.44 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1_{Glc}), 4.09 (dd, 1H, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 3.1 Hz, H-3_{Gal}), 3.96-3.83 (m, 5H, H-4_{Gal}, -C$\underline{H}_2$O-, H-$6a_{Glc}$, H-8_{Neu}, H-5_{Neu}), 3.79-3.68 (m, 6H, H-$6b_{Glc}$, -C$\underline{H}_2$O-, H-5_{Gal}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-$9a_{Neu}$, H-4_{Neu}), 3.65-3.54 (m, 7H, H-6_{Neu}, H-3_{Glc}, H-2_{Gal}, H-5_{Glc}, H-4_{Glc}, H-$9b_{Neu}$, H-7_{Neu}), 3.37 (br s, 2H, -CH_2NH-), 3.30 (dd, 1H, $J_{2,1} \approx J_{2,3} \approx 7.1$ Hz, H-2$_{Glc}$), 2.76 (dd, 1H, $J_{3e,3a} = 12.2$ Hz, $J_{3e,4} = 3.4$ Hz, H-3e$_{Neu}$), 2.02 (s, 3H, C(O)CH$_3$), 1.80 (dd, 1H, $J_{3a,3e} \approx J_{3a,4} \approx 12.4$ Hz); ^{13}C NMR (125 MHz, D$_2$O): δ_C 175.9, 174.6, 159.3, 129.7, 129.3, 128.6, 103.6, 103.2, 100.7, 79.1, 76.4, 76.0, 75.6, 75.2, 73.8, 73.7, 72.6, 70.2, 69.9, 69.2, 69.0, 68.4, 67.8, 63.5, 61.9, 60.9, 52.6, 41.4, 20.5, 22.9. ESI HRMS calc'd for $C_{33}H_{50}N_2O_{21}Na$: 833.2798. Found: 833.2798.

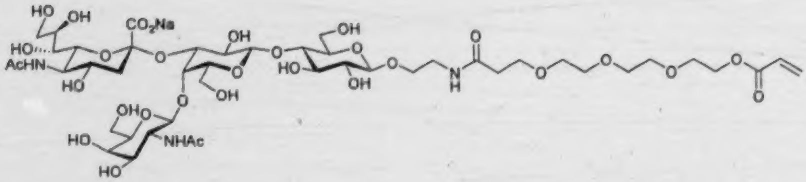


O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-2-*N*-(benzyloxycarbonyl)-aminoethanol (74). The acceptor **73** (50.1 mg; 60.2 μmol) and UDP-*N*-acetylglucosamine disodium salt (48.0 mg; 73.8 μmol; 1.5 eq) were combined in a Falcon tube. UDP-*N*-acetylglucosamine 4-epimerase (2.0 mL; ~ 2.6 U) in 0.2 M NaCl, 1.0 mM EDTA, 20 mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 and *N*-acetylgalactosaminyl transferase (5.9 mL; ~ 3.5 U) were added to the mixture followed by 96 μL of 0.5 M MgCl$_2$ (6.0 mM in reaction) and alkaline phosphatase (20 μL; ~ 20 U). The reaction was gently tumbled for 16 hr until the reaction was completed as observed by silica gel TLC (EtOAC-MeOH-H$_2$O-AcOH; 6:3:3:2). The reaction mixture was applied to a Sep-Pak (C18; 5 g). Elution with a gradient of water-methanol afforded **74**, which was further purified by size exclusion chromatography (Biogel P-4 resin; 75 cm × 2 cm) and eluted with water to yield **74** (48.8 mg; 47.1 μmol; 78 %): ^{1}H NMR (600 MHz, D$_2$O): δ_H 7.44-7.37 (m, 5H, Ar-H), 5.11 (s, 2H, ArCH$_2$O-), 4.74 (d, 1H, $J_{1,2} = 7.3$ Hz, H-1$_{GalNAc}$), 4.50 (d, 1H, $J_{1,2} = 8.0$ Hz, H-1$_{Gal}$), 4.44 (br d, 1H, $J_{1,2} = 7.6$ Hz, H-1$_{Glc}$), 4.14 (ddd, 1H, $J = 9.8$ Hz, $J \approx J \approx 3.0$ Hz, -CH$_2$O-), 4.10 (d, 1H, $J_{4,3} = 2.9$ Hz, H-4$_{Gal}$), 3.92-3.56 (m, 19H, H-4$_{GalNAc}$, H-2$_{GalNAc}$, H-9a$_{Neu}$, H-5$_{Neu}$, H-6b$_{Glc}$, H-4$_{Neu}$, H-5$_{Gal}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{GalNAc}$, H-6a$_{GalNAc}$, H-6b$_{GalNAc}$, H-8$_{Neu}$, H-3$_{GalNAc}$, H-3$_{Glc}$, H-9b$_{Neu}$, H-4$_{Glc}$, H-7$_{Neu}$, H-5$_{Glc}$), 3.49 (dd, 1H, $J_{6,5} = 10.0$ Hz, $J_{6,7} = 2.0$ Hz, H-6$_{Neu}$), 3.37-3.34 (m, 3H, H-2$_{Gal}$, -CH$_2$NH-), 3.28 (dd, 1H, $J_{2,1} \approx J_{2,3} \approx 8.4$ Hz, H-2$_{Glc}$), 2.66 (dd, 1H, $J_{3e,3a} = 12.6$ Hz, $J_{3e,4} = 4.4$ Hz, H-

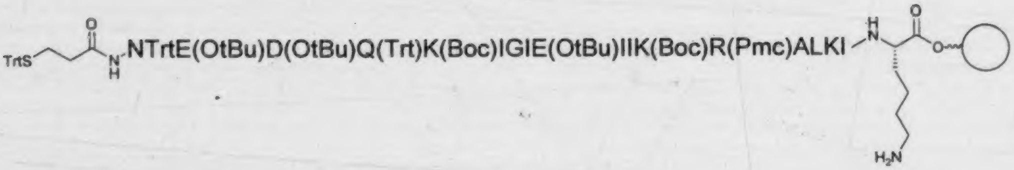
$3e_{\text{Neu}}$), 2.03, 2.01 (2 × s, 2 × 3H, 2 × C(O)$\underline{\text{CH}_3}$), 1.92 (dd, 1H, $J_{3a,3e}$ = 11.8 Hz, $J_{3a,4}$ = 11.4 Hz, H-$3a_{\text{Neu}}$); ^{13}C NMR (125 MHz, D_2O) : δ_C 175.9, 175.7, 174.8, 159.3, 129.7 (4C), 129.3, 128.6, 103.6, 103.5, 103.2, 102.4, 79.6, 78.2, 75.8, 75.4, 75.3, 75.1, 74.1, 73.8, 73.3, 72.3, 71.0, 70.1, 69.7, 69.1, 68.8, 68.0, 63.8, 62.0, 61.4, 61.0, 53.2, 52.5, 41.1, 37.9, 23.5, 22.9. ESI HRMS calc'd for $C_{41}H_{62}N_3O_{26}$: 1012.3627. Found: 1012.3629.



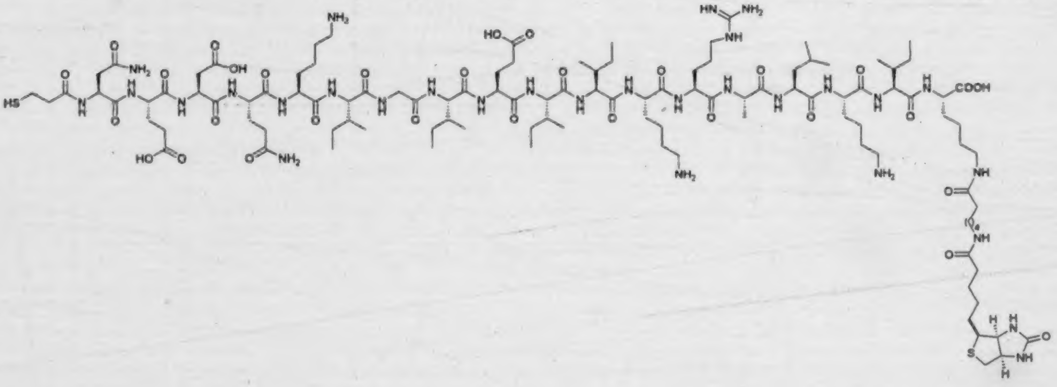
O-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-O-β-D-galactopyranosyl-(1→4)]-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-2-aminoethanol (acetic acid salt) (75). Compound **74** (40 mg; 38.6 μmol) was dissolved in dry methanol (5 mL). Palladium hydroxide (20 wt. %Pd on carbon, Degussa type) (5 mg) was added followed by 5 drops of acetic acid. The flask was evacuated and the solution saturated with hydrogen gas. The reaction mixture was stirred under a hydrogen atmosphere for 16 hr. After this time, the mixture was filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 μM) and the solvent was evaporated. The residue was lyophilized to obtain **75** as a white solid (31.8 mg; 35.2 μmol; 91%): ^{1}H NMR (500 MHz, D_2O): δ_H 4.74 (d, overlapped with HOD, 1H, H-1_{GalNAc}), 4.53 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Gal}), 4.52 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1_{Glc}), 4.15-4.09 (m, 3H, H-3_{Gal}, -C$\underline{H_2}$O-, H-4_{Gal}), 4.00-3.90 (m, 5H, -CH_2O-, H-$6a_{\text{Glc}}$, H-$9a_{\text{Neu}}$, H-4_{GalNAc}, H-2_{GalNAc}), 3.87-3.70 (m, 8H, H-$6b_{\text{Glc}}$, H-5_{Neu}, H-4_{Neu}, H-5_{Gal}, H-8_{Neu}, H-5_{GalNAc}, H-$9b_{\text{Neu}}$, H-3_{GalNAc}), 3.69-3.58 (m, 8H, H-$6a_{\text{Gal}}$, H-$6b_{\text{Gal}}$, H-$6a_{\text{GalNAc}}$, H-$6b_{\text{GalNAc}}$, H-3_{Glc}, H-4_{Glc}, H-7_{Neu}, H-5_{Glc}), 3.48 (dd, 1H, $J_{6,5}$ = 10.0 Hz, $J_{6,7}$ = 2.0 Hz, H-6_{Neu}), 3.38-3.34 (m, 2H, H-2_{Gal}, H-2_{Glc}), 3.27 (t, 2H, J = 5.1 Hz, -CH_2NH-), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.6 Hz, $J_{3e,4}$ = 4.4 Hz, H-$3e_{\text{Neu}}$), 2.03, 2.02 (2 × s, 2 × 3H, 2 × C(O)$\underline{\text{CH}_3}$), 1.91 (dd, 1H, $J_{3a,3e} \approx J_{3a,4} \approx$ 11.8 Hz, H-$3a_{\text{Neu}}$); ^{13}C NMR (125 MHz, D_2O) : δ_C 175.9, 175.7, 174.9, 103.6, 103.5, 102.8, 102.5, 79.3, 78.0, 75.7, 75.6, 75.2, 75.1, 74.9, 74.0, 73.5, 73.2, 72.1, 70.9, 69.6, 68.9, 68.7, 66.7, 63.7, 62.0, 61.5, 60.9, 53.2, 52.5, 40.3, 37.9, 23.5, 22.9. ESI HRMS calc'd for $C_{33}H_{56}N_3O_{24}$: 878.3259. Found: 878.3260.



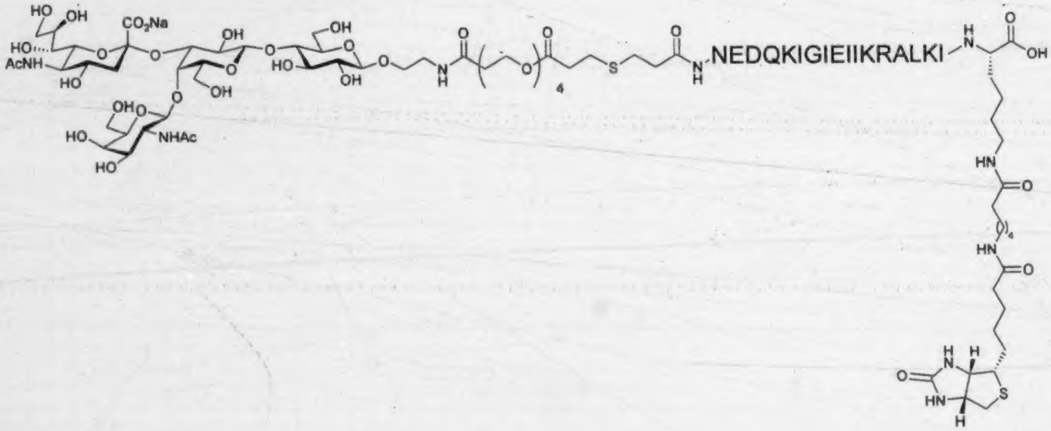
***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-2-*N*-(14-oxo-4,7,10,13-tetrahexadec-15-enoate)-aminoethanol (76)**: A solution of GM_2-amine **75** (5.1 mg, 5.8 μmol) in aqueous borate buffer (1 mL; 0.02 M, pH 8.1) was added to heterobifunctional linker **71** (4.6 mg, 12 μmol, 2.1 eq). The mixture was vortexed for 5 min and subsequently tumbled for 35 min at room temperature. After this time, the reaction mixture was filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 μM), and injected into a preparative HPLC system. The desired GM_2 analogue **76** was isolated using an acetonitrile/water gradient and obtained as colorless lyophilisate after freeze-drying (5.2 mg, 4.6 μmol, 79 %): tR=27.0 min (Phenomenex Jupiter C12, acetonitrile/water + 0.1 % TFA 5:95→50:50, 65 min). ^{1}H NMR (600 MHz, D2O): δ_H 6.46-6.42 (m, 1 H, H-2$c_{acrylate}$), 6.21 (dd, 1 H, $J_{1,2t}$ = 17.4 Hz, $J_{1,2c}$ = 10.8 Hz, H-$1_{acrylate}$), 6.01-5.98 (m, 1 H, H-2$t_{acrylate}$), 4.71 (d, 1 H, $J_{1,2}$ = 8.6 Hz, H-1_{GalNAc}), 4.51 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1_{Gal}), 4.48 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1_{Glc}), 4.36-4.33 (m, 2 H, $CH_2OC(O)$-), 4.14 (dd, 1 H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 3.4 Hz, H-3_{Gal}), 4.09 (d, 1 H, $J_{4,3}$ = 2.9 Hz, H-4Gal), 3.98-3.93 (m, 2 H, -OCH_2CH_2N-, H-6a_{Glc}), 3.92-3.87 (m, 1 H, H-2_{GalNAc}), 3.86 (dd, 1 H, $J_{9a,9b}$ = 12.0 Hz, $J_{9a,8}$ = 2.2 Hz, H-9a_{Neu}), 3.83-3.55 (m, 29 H, 11-CH_2 linker, H-5_{Neu}, -OCH_2CH_2N-, H-4_{Neu}, H-4_{GalNAc} , H-6b_{Glc}, H-3_{GalNAc}, H-4_{Glc}, H-3_{Glc}, 5 × CH_2 linker, H-9b_{Neu}, H-5_{Glc}, H-7_{Neu}, H-8_{Neu}, H-5_{GalNAc}, H-6GalNAc, H-5_{Gal}, H-6_{Gal}), 3.49 (dd, 1 H, , $J_{6,5}$ = 10.1 Hz, $J_{6,7}$ = 1.9 Hz, H-6_{Neu}), 3.48-3.38 (m, 2 H), 3.36 (dd, 1 H, $J_{2,1}$ = 8.1 Hz, $J_{2,3}$ = 9.7 Hz, H2_{Gal}), 3.31 (dd, 1 H, $J_{2,1}$ = $J_{2,3}$ = 8.4 Hz, H-2_{Glc}), 2.66 (dd, 1 H, $J_{3e,3a}$ = 12.6 Hz, $J_{3,4}$ = 4.7 Hz, H-3e_{NeuNAc}), 2.53 (t, 2 H, $J_{2,3}$ = 6.2 Hz, 2-CH_2 linker), 2.02, 2.00 (2 × s, 2 × 3 H, -N(O)CH3), 1.92 (dd, 1 H, $J_{3a,3e}$ = $J_{3a,4}$ = 12.0 Hz, H-3a_{Neu}); ^{13}C NMR (150 MHz, D2O, chemical shifts taken from HSQC, HMBC): δ_C 133.6, 128.4, 103.7, 103.2, 38.2. MALDI TOF MS (positive, DHB) calc'd for $C_{45}H_{75}N_3O_{30}Na$: 1161.1. Found: 1160.9.



TrtS-(CH$_2$)$_2$-Asn(Trt)-Glu(OtBu)-Asp(OtBu)-Gln(Trt)-Lys(Boc)-Ile-Gly-Ile-Glu(OtBu)-Ile-Ile-Lys(Boc)-Arg(Pmc)-Ala-Leu-Lys(Boc)-Ile-Lys-TentaGel R (77): The p458m amino acid sequence was assembled on solid phase starting from Fmoc-Lys(ivDde)-OH functionalized TentaGel R resin (0.1 mmol, substitution: 0.17 mmol g-1) in an ABI 433 A peptide synthesizer using Fmoc chemistry. Upon completion of the peptide sequence and removal of the N-terminal Fmoc group, *S*-trityl-β-mercaptopropionic acid (2 × 348.4 mg, 1 mmol) was attached in a standard double coupling, and the resin was extensively washed with NMP and dichloromethane. The resin-bound peptide was transferred into a Merrifield glass reactor, washed with DMF (3 × 5 mL) and treated with a 3.5 % solution of hydrazine monohydrate in DMF (3 mL) for 5 min. The resin was filtered and the hydrazine treatment, repeated four more times. The progress of the reaction was followed by monitoring the UV absorbance of an aliquot of the deprotection solutions at λ = 290 nm. Subsequently, the resin was washed with DMF (5 × 5 mL) and dichloromethane (3 × 5 mL), and dried *in vacuo* to afford 768 mg of the selectively deprotected resin-bound peptide **77**.



HS-(CH2)$_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-Lys(biotin)-OH (HS-p458m-bio) (78): A portion of resin 77 (268 mg, max. 0.033 mmol) was placed into the ABI A433 peptide synthesizer, and washed with dichloromethane and NMP. The biotinylation at the selectively deprotected lysine residue was carried out in a double coupling with extended coupling times of 1 h each employing biotin (2 × 244 mg, 2 × 1 mmol) and the standard activating conditions of the synthesizer (1 mmol HBTU/HOBt, 2 mmol DIPEA in DMF). After extensive washing with NMP and dichloromethane, the resin was treated in a Merrifield reactor with a cleavage cocktail consisting of TFA (7.5 mL), water (0.45 mL) and TIS (0.45 mL) for 2.5 h. Subsequently, the resin was filtered and washed with TFA (3 × 3 mL). The combined filtrates were concentrated *in vacuo* and co-evaporated with toluene (3 × 15 mL). The peptide was precipitated by addition of cold (0 °C) diethyl ether (10 mL) to furnish a white solid, which was repeatedly washed with diethyl ether. The crude product was purified by preparative RP-HPLC (Phenomenex Luna C_{18}(2), acetonitrile/water + 0.1 % TFA, 25:75→35:65 + 0.1 % TFA, 65 min, λ=212 nm, 32 min) to afford biotinylated building block **78** (30.3 mg, 0.0125 mmol, 38 %) as a white solid after lyophilization: Analytical HPLC: tR=17.1 min (Phenomenex Luna C_{18}(2), acetonitrile/water + 0.1 % TFA 25:75 → 50:50, 25 min). MALDI TOF MS (positive, sinapinic acid): m/z: calc'd for $C_{107}H_{188}N_{29}O_{30}S_2$: 2424.94. Found: 2424.99.



GM$_2$-(CH$_2$)$_3$S(CH$_2$)$_2$NHCO(CH$_2$CH$_2$O)$_4$CO(CH$_2$)$_2$S(CH$_2$)$_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-Lys(Biotin)-OH (GM2-p458 m-bio) (66): In a Falcon tube GM2-acrylate building block **76** (2.2 mg, 1.93 µmol) and HS-p458m-bio **78** (4.7 mg, 1.94 µmol) were combined in aqueous Tris HCl buffer (3 mL; 0.05 M Tris, 1 mM EDTA; pH 8.9). The mixture was sonicated for 10 min and subsequently tumbled for 6 h under argon. The solution was filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 µM), and the biotinylated glycoconjugate **66** (4.8 mg, 1.35 µmol, 70 %) was isolated by preparative RP-HPLC (Phenomenex Luna C_{18}(2), acetonitrile/water + 0.1 % TFA, 25:75→35:65 + 0.02 % TFA, 65 min, λ = 212 nm, tR=20.8 min) and obtained as a colorless solid after lyophilization: Analytical HPLC: tR=13.8 min (Phenomenex Luna C_{18}(2), acetonitrile/water + 0.02 % TFA 25:55→35:65, 25 min). MALDI TOF MS (positive, sinapinic acid): m/z: calc'd for $C_{152}H_{263}N_{32}O_{60}S_2$: 3560.80. Found: 3561.61.

References

1. Geremia, R. A.; Samain, E. *Carbohydrate-based Drug Discovery* **2003**, *1*, 845-860.
2. Dwek, R. A. *Biochem. Soc. Trans.* **1995**, *23*, 1-25.
3. Miller, D. J.; Ax, R. L. *Mol. Reprod. Dev.* **1990**, *26*, 184-198.
4. Diekman, A. B. *Cellular and Molecular Life Sciences* **2003**, *60*, 298-308.
5. Ioffe, E.; Stanley, P. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 728-732.
6. Schachter, H. *Curr. Opin. Struct. Biol.* **2004**, *14*, 607-616.
7. Haltiwanger, R. S.; Lowe, J. B. *Annu. Rev. Biochem.* **2004**, *73*, 491-537.
8. Lasky, L. A. *Annu. Rev. Biochem.* **1995**, *64*, 113-139.
9. Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. *Science* **2001**, *291*, 2370-2376.
10. Masuoka, J. *Clin. Microbiol. Rev.* **2004**, *17*, 281.
11. Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357-2364.
12. Taylor, M. E.; Drickamer, K. In *Introduction to Glycobiology;* Oxford University Press: 2003; pp 207.
13. Marshall, R. D. *Annu. Rev. Biochem.* **1972**, *41*, 673.
14. Gavel, Y.; Vonheijne, G. *Protein Eng.* **1990**, *3*, 433-442.
15. Harris, R. J.; Leonard, C. K.; Guzzetta, A. W.; Spellman, M. W. *Biochemistry (N.Y.)* **1991**, *30*, 2311-2314.
16. Holt, G. D.; Hart, G. W. *J. Biol. Chem.* **1986**, *261*, 8049-8057.
17. Finne, J.; Krusius, T.; Margolis, R. K.; Margolis, R. U. *J. Biol. Chem.* **1979**, *254*, 295-300.
18. Hakomori, S. I.; Igarashi, Y. *Adv. Lipid Res. Vol 25* **1993**, *25*, 147-162.
19. Stults, C. L. M.; Sweeley, C. C.; Macher, B. A. *Meth. Enzymol.* **1989**, *179*, 167-214.
20. Schwarzmann, G.; Sandhoff, K. *Biochemistry (N.Y)* **1990**, *29*, 10865-10871.

21. Hakomori, S. *Arch. Biochem. Biophys.* **2004**, *426*, 173-181.

22. Gabius, H. J.; Siebert, H. C.; Andre, S.; Jimenez-Barbero, J.; Rudiger, H. *Chembiochem* **2004**, *5*, 741-764.

23. Horan, N.; Yan, L.; Isobe, H.; Whitesides, G. M.; Kahne, D. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 11782-11786.

24. Mammen, M.; Choi, S. K.; Whitesides, G. M. *Angew. Chem. Int. Edit.* **1998**, *37*, 2755-2794.

25. Whitfield, D. M.; Douglas, S. P. *Glycoconj. J.* **1996**, *13*, 5-17.

26. Boons, G. J. *Contemp. Org. Synthesis* **1996**, *3*, 173-200.

27. Davis, B. G. *J. Chem. Soc.Perkin Trans. 1* **2000**, 2137-2160.

28. Paulsen, H. *Angew. Chem. Int. Edit.* **1982**, *21*, 155-173.

29. Garegg, P. J. *Adv. Carbohydr. Chem. Biochem., Vol 52* **1997**, *52*, 179-205.

30. Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem., Vol 50* **1994**, *50*, 21-123.

31 Stick, R. V. In *Carbohydrates: The Sweet Molecules of Life;* Academic Press: San Diego, 2001; , pp 256.

32. Demchenko, A. V. *Letters in Organic Chemistry* **2005**, *2*, 580-589.

33. Demchenko, A. V. *Curr. Org. Chem.* **2003**, *7*, 35-79.

34. Nukada, T.; Berces, A.; Zgierski, M. Z.; Whitfield, D. M. *J. Am. Chem. Soc.* **1998**, *120*, 13291-13295.

35. Schmidt, R. R.; Behrendt, M.; Toepfer, A. *Synlett* **1990**, 694-696.

36. Ratcliffe, A. J.; Fraserreid, B. *J. Chem. Soc. Perkin Trans. 1* **1990**, 747-750.

37. Ito, Y.; Ogawa, T. *Tetrahedron Lett.* **1988**, *29*, 1061-1064.

38. Lemieux, R. U.; Hendriks, K. B.; Stick, R. V.; James, K. *J. Am. Chem. Soc.* **1975**, *97*, 4056-4062.

39 Lemieux, R. U.; Hayimi, J. L. *Can. J. Chem.* **1965**, *43*, 2162.

40 Lowary, T. L.; Eichler, E.; Bundle, D. R. *J. Org. Chem.* **1995**, *60*, 7316-7327.

41. Paulsen, H.; Lebuhn, R.; Lockhoff, O. *Carbohydr. Res.* **1983**, *103*, C7-C11.

42. Alais, J.; David, S. *Carbohydr. Res.* **1990**, *201*, 69-77.

43. Furstner, A.; Konetzki, I. *Tetrahedron Lett.* **1998**, *39*, 5721-5724.

44. Auge, C.; Warren, C. D.; Jeanloz, R. W.; Kiso, M.; Anderson, L. *Carbohydr. Res.* **1980**, *82*, 85-95.

45 Barresi, F.; Hindsgaul, O. *J. Am. Chem. Soc.* **1991**, *113*, 9376-9377.

46 Stork, G.; Kim, G. *J. Am. Chem. Soc.* **1992**, *114*, 1087-1088.

47. Joe, M.; Bai, Y.; Nacario, R. C.; Lowary, T. L. *J. Am. Chem. Soc.* **2007**, *129*, 9885-9901.

48. Kren, V.; Thiem, J. *Chem. Soc. Rev.* **1997**, *26*, 463-473.

49. Gambert, U.; Thiem, J. *Glycoscience Synthesis of Oligosaccharides and Glycoconjugates* **1997**, *186*, 21-43.

50. Leloir, L. F. *Science* **1971**, *172*, 1299.

51. Ernst, B.; Oehrlein, R. *Glycoconj. J.* **1999**, *16*, 161-170.

52. Qian, X. P.; Sujino, K.; Otter, A.; Palcic, M. M.; Hindsgaul, O. *J. Am. Chem. Soc.* **1999**, *121*, 12063-12072.

53. Sujino, K.; Uchiyama, T.; Hindsgaul, O.; Seto, N. O. L.; Wakarchuk, W. W.; Palcic, M. M. *J. Am. Chem. Soc.* **2000**, *122*, 1261-1269.

54. Gijsen, H. J. M.; Qiao, L.; Fitz, W.; Wong, C. H. *Chem. Rev.* **1996**, *96*, 443-473.

55. Wong, C. H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 5416-5418.

56. Ichikawa, Y.; Lin, Y. C.; Dumas, D. P.; Shen, G. J.; Garciajunceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C. H. *J. Am. Chem. Soc.* **1992**, *114*, 9283-9298.

57. Chen, X.; Liu, Z. Y.; Zhang, J. B.; Zhang, W.; Kowal, P.; Wang, P. G. *Chembiochem* **2002**, *3*, 47-53.

58. Jakeman, D. L.; Withers, S. G. *Trends in Glycoscience and Glycotechnology* **2002**, *14*, 13-25.

59. Williams, S. J.; Withers, S. G. *Aust. J. Chem.* **2002**, *55*, 3-12.

60 Rye, C. S.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2000**, *4*, 573-580.

61 Williams, S. J.; Withers, S. G. *Carbohydr. Res.* **2000**, *327*, 27-46.

62. Ruffing, A.; Chen, R. R. *Microb. Cell Fact.* **2006**, *5*.

63. Endo, T.; Koizumi, S. *Curr. Opin. Struct. Biol.* **2000**, *10*, 536-541.

64. Randriantsoa, M.; Drouillard, S.; Breton, C.; Samain, E. *FEBS Lett.* **2007**, *581*, 2652-2656.

65. Drouillard, S.; Driguez, H.; Samain, E. *Angew. Chem. Int. Edit.* **2006**, *45*, 1778-1780.

66. Dumon, C.; Bosso, C.; Utille, J. P.; Heyraud, A.; Samain, E. *Chembiochem* **2006**, *7*, 359-365.

67. Antoine, T.; Heyraud, A.; Bosso, C.; Samain, E. *Angew. Chem. Int. Edit.* **2005**, *44*, 1350-1352.

68. Fort, S.; Birikaki, L.; Dubois, M. P.; Antoine, T.; Samain, E.; Driguez, H. *Chem. Commun.* **2005**, 2558-2560.

69. Antoine, T.; Priem, B.; Heyraud, A.; Greffe, L.; Gilbert, M.; Wakarchuk, B. W.; Lam, J. S.; Samain, E. *Chembiochem* **2003**, *4*, 406-412.

70. Samain, E.; Chazalet, V.; Geremia, R. A. *J. Biotechnol.* **1999**, *72*, 33-47.

71. Koeller, K. M.; Wong, C. H. *Nat. Biotechnol.* **2000**, *18*, 835-841.

72. Seeberger, P. H.; Werz, D. B. *Nature* **2007**, *446*, 1046-1051.

73. Goldsby, R. A.; Kindt, T. J.; Osborne, B. A.; Kuby, J. In *Immunology;* W. H. Freeman: New York, **2003**, pp 551.

74. Feizi, T. *Nature* **1985**, *314*, 53-57.

75. Hakomori, S. *Cancer Res.* **1996**, *56*, 5309-5318.

76. Kim, Y. J.; Varki, A. *Glycoconj. J.* **1997**, *14*, 569-576.

77. Danishefsky, S. J.; Allen, J. R. *Angew. Chem. Int. Edit.* **2000**, *39*, 836-863.

78. Treves, A. J.; Carnaud, C.; Trainin, N.; Feldman, M.; Cohen, I. R. *Eur. J. Immunol.* **1974**, *4*, 722-727.

79. Fujimoto, S.; Greene, M. I.; Sehon, A. H. *J. Immunol.* **1976**, *116*, 791-799.

80. Bodmer, W. F.; Browning, M. J.; Krausa, P.; Rowan, A.; Bicknell, D. C.; Bodmer, J. G. *Ann. N. Y. Acad. Sci.* **1993**, *690*, 42-49.

81. Stevenson, F. K.; Rice, J. *Eur. J. Immunol.* **2006**, *36*, 1070-1073.

82. Svennerholm, L. *J. Neurochem* **1963**, *10*, 613.

83. Seifert, H. *Klin. Wachenschr* **1966**, *44*, 469-470.

84. Fredman, P. *Adv. Lipid. Res.* **1993**, *25*, 213-234.

85. Hakomori, S. I. *Adv. Cancer Res.* **1989**, *52*, 257-331.

86. Fredman, P.; Hedberg, K.; Brezicka, T. *Biodrugs* **2003**, *17*, 155-167.

87. Karlsson, G.; Mansson, J. E.; Wikstrand, C. *Biochim. Biophys. Acta* **1990**, *1043*, 267-272.

88. Livingston, P. O. *Immunol. Rev.* **1995**, *145*, 147-166.

89. Livingston, P. O.; Wong, G. Y. C.; Adluri, S.; Tao, Y.; Padavan, M.; Parente, R.; Hanlon, C.; Calves, M. J.; Helling, F.; Ritter, G.; Oettgen, H. F.; Lloyd, J. O. *J. Clin. Oncol.* **1994**, *12*, 1036-1044.

90. Zhang, H.; Zhang, S. L.; Cheung, N. K. V.; Ragupathi, G.; Livingston, P. O. *Cancer Res.* **1998**, *58*, 2844-2849.

91. Hakomori, S.; Zhang, Y. M. *Chem. Biol.* **1997**, *4*, 97-104.

92. Livingston, P. O.; Watanabe, T.; Shiku, H.; Houghton, A. N.; Albino, A.; Takahashi, T.; Resnick, L. A.; Michitsch, R.; Pinsky, C. M.; Oettgen, H. F.; Old, L. J. *Int. J. Cancer* **1982**, *30*, 413-422.

93. Livingston, P. O.; Takeyama, H.; Pollack, M. S.; Houghton, A. N.; Albino, A.; Pinsky, C. M.; Oettgen, H. F.; Old, L. J. *Int. J. Cancer* **1983**, *31*, 567-575.

94 Tai, T.; Cahan, L. D.; Tsuchida, T.; Saxton, R. E.; Irie, R. F.; Morton, D. L. *Int. J. Cancer* **1985**, *35*, 607-612.

95. Livingston, P. O.; Calves, M. J.; Natoli, E. J. *J. Immunol.* **1987**, *138*, 1524-1529.

96. Livingston, P. O.; Natoli, E. J.; Calves, M. J.; Stockert, E.; Oettgen, H. F.; Old, L. J. *Proc. Natl. Acad. Sci. U. S. A.* **1987**, *84*, 2911-2915.

97. Helling, F.; Shang, A.; Calves, M.; Zhang, S. L.; Ren, S. L.; Yu, R. K.; Oettgen, H. F.; Livingston, P. O. *Cancer Res.* **1994**, *54*, 197-203.

98. Chapman, P. B.; Morrissey, D. M.; Panageas, K. S.; Hamilton, W. B.; Zhan, C.; Destro, A. N.; Williams, L.; Israel, R. J.; Livingston, P. O. *Clin. Cancer Res.* **2000**, *6*, 874-879.

99. Livingston, P. O.; Ragupathi, G.; Musselli, C. *J. Clin. Immunol.* **2000**, *20*, 85-93.

100. Nudelman, E.; Hakomori, S.; Kannagi, R.; Levery, S.; Yeh, M. Y.; Hellstrom, K. E.; Hellstrom, I. *J. Biol. Chem.* **1982**, *257*, 2752-2756.

101. Itonori, S.; Hidari, K.; Sanai, Y. *Glycoconj. J.* **1989**, *6*, 551-560.

102. Hakomori, S.; Igarashi, Y. *J. Biochem.* **1995**, *118*, 1091-1103.

103. Chatterjee, S.; Wei, H. M. *Recognition of Carbohydrates in Biological Systems, Part B: Specific Applications* **2003**, *363*, 300-312.

104. Dyatlovitskaya, E. V.; Kandyba, A. G. *Russ. J. Bioorg. Chem.* **2004**, *30*, 201-206.

105. Bremer, E. G. *Cell Lipids* **1994**, *40*, 387-411.

106. Hakomori, S. I. *Annu. Rev. Biochem.* **1981**, *50*, 733-764.

107. Hakomori, S. I. *J. Biol. Chem.* **1990**, *265*, 18713-18716.

108. Ohanian, J.; Ohanian, V. *Cell. Mol. Life Sci.* **2001**, *58*, 2053-2068.

109. Bektas, M.; Spiegel, S. *Glycoconj. J.* **2003**, *20*, 39-47.

110. Bieberich, E. *Glycoconj. J.* **2004**, *21*, 315-327.

111. Hakomori, S. *Glycoconj. J.* **2000**, *17*, 627-647.

112. Vankar, Y. D.; Schmidt, R. R. *Chem. Soc. Rev.* **2000**, *29*, 201-216.

113. Rai, A. N.; Basu, A. *Org. Lett.* **2004**, *6*, 2861-2863.

114. Khiar, N.; Singh, K.; Garcia, M.; Martin-Lomas, M. *Tetrahedron Lett.* **1999**, *40*, 5779-5782.

115. Compostella, F.; Franchini, L.; Panza, L.; Prosperi, D.; Ronchetti, F. *Tetrahedron* **2002**, *58*, 4425-4428.

116. He, L. L.; Byun, H. S.; Bittman, R. *J. Org. Chem.* **2000**, *65*, 7627-7633.

117. Kobayashi, S.; Hayashi, T.; Kawasuji, T. *Tetrahedron Lett.* **1994**, *35*, 9573-9576.

118. Schauer, R.; Klem, S.; Reuter, G.; Roggentin, P.; Shaw, L. In Rosenberg, A., Ed.; Biology of The Sialic Acids; Plenum Press: New York, 1995; .

119. Boons, G. J.; Demchenko, A. V. *Chem. Rev.* **2000**, *100*, 4539-4565.

120. Ishida, H.; Kiso, M. *Trends in Glycosci. and Glyc.* **2001**, *13*, 57-64.

121. Meindl, P.; Tuppy, H. *Monatshefte Fur Chemie Und Verwandte Teile Anderer Wissenschaften* **1965**, *96*, 802-&.

122. Schwarz, J. B.; Kuduk, S. D.; Chen, X. T.; Sames, D.; Glunz, P. W.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 2662-2673.

123. Toshima, K.; Tatsuta, K. *Chem. Rev.* **1993**, *93*, 1503-1531.

124. Marra, A.; Sinay, P. *Carbohydr. Res.* **1989**, *187*, 35-42.

125. Martin, T. J.; Schmidt, R. R. *Tetrahedron Lett.* **1992**, *33*, 6123-6126.

126. Kondo, H.; Ichikawa, Y.; Wong, C. H. *J. Am. Chem. Soc.* **1992**, *114*, 8748-8750.

127. Kanie, O.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1988**, *7*, 501-506.

128. Hasegawa, A.; Nagahama, T.; Ohki, H.; Hotta, K.; Ishida, H.; Kiso, M. *J. Carbohydr. Chem.* **1991**, *10*, 493-498.

129. Braccini, I.; Derouet, C.; Esnault, J.; Dupenhoat, C. H.; Mallet, J. M.; Michon, V.; Sinay, P. *Carbohydr. Res.* **1993**, *246*, 23-41.

130. Murase, T.; Ishida, H.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1989**, *188*, 71-80.

131. Murase, T.; Ishida, H.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1988**, *184*, C1-C4.

132. Murase, T.; Kameyama, A.; Kartha, K. P. R.; Ishida, H.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1989**, *8*, 265-283.

133. Ress, D. K.; Linhardt, R. J. *Current Organic Synthesis* **2004**, *1*, 31-46.

134. Okamoto, K.; Kondo, T.; Goto, T. *Tetrahedron Lett.* **1986**, *27*, 5233-5236.

135. Okamoto, K.; Kondo, T.; Goto, T. *Tetrahedron* **1987**, *43*, 5919-5928.

136. Okamoto, K.; Kondo, T.; Goto, T. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 637-643.

137. Okamoto, K.; Kondo, T.; Goto, T. *Tetrahedron* **1987**, *43*, 5909-5918.

138. Ito, Y.; Ogawa, T. *Tetrahedron Lett.* **1988**, *29*, 3987-3990.

139. Ito, Y.; Ogawa, T. *Tetrahedron* **1990**, *46*, 89-102.

140. Ito, Y.; Ogawa, T. *Tetrahedron Lett.* **1987**, *28*, 6221-6224.

141. Takahashi, T.; Tsukamoto, H.; Yamada, H. *Tetrahedron Lett.* **1997**, *38*, 8223-8226.

142. Palcic, M. M. *Curr. Opin. Biotechnol.* **1999**, *10*, 616-624.

143. Qian, X. P.; Sujino, K.; Palcic, M. M.; Ratcliffe, R. M. *J. Carbohydr. Chem.* **2002**, *21*, 911-942.

144. Hanson, S.; Best, M.; Bryan, M. C.; Wong, C. H. *Trends Biochem. Sci.* **2004**, *29*, 656-663.

145. Daines, A. M.; Maltman, B. A.; Flitsch, S. L. *Curr. Opin. Chem. Biol.* **2004**, *8*, 106-113.

146. Rowan, A. S.; Hamilton, C. J. *Nat. Prod. Rep.* **2006**, *23*, 412-443.

147. Seibel, J.; Jordening, H. J.; Buchholz, K. *Biocatalysis Biotransformation* **2006**, *24*, 311-342.

148. Peterson, M. A.; Polt, R. *J. Org. Chem.* **1993**, *58*, 4309-4314.

149. Schmidt, R. R.; Zimmermann, P. *Angew. Chem. Int. Edit.* **1986**, *25*, 725-726.

150. Ofsson, B.; Somfai, P. *J. Org. Chem.* **2003**, *68*, 2514-2517.

151. Rai, A. N.; Basu, A. *Org. Lett.* **2004**, *6*, 2861-2863.

152. Torssell, S.; Somfai, P. *Org. Biomol. Chem.* **2004**, *2*, 1643-1646.

153. Bundle, D. R. Ling C.C. Zhang, P. PCT/CA03/00832, 2003.

154. Auge, C.; Gautheron, C.; Pora, H. *Carbohydr. Res.* **1989**, *193*, 288-293.

155. Herrmann, G. F.; Ichikawa, Y.; Wandrey, C.; Gaeta, F. C. A.; Paulson, J. C.; Wong, C. H. *Tetrahedron Lett.* **1993**, *34*, 3091-3094.

156. Thiem, J.; Treder, W. *Angew. Chem. Int. Edit.* **1986**, *25*, 1096-1097.

157. Blixt, O.; Allin, K.; Pereira, L.; Datta, A.; Paulson, J. C. *J. Am. Chem. Soc.* **2002**, *124*, 5739-5746.

158. Auge, C.; Fernandezfernandez, R.; Gautheron, C. *Carbohydr. Res.* **1990**, *200*, 257-268.

159. Sabesan, S.; Paulson, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 2068-2080.

160. Unverzagt, C.; Kunz, H.; Paulson, J. C. *J. Am. Chem. Soc.* **1990**, *112*, 9308-9309.

161. Gilbert, M.; Bayer, R.; Cunningham, A. M.; Defrees, S.; Gao, Y. H.; Watson, D. C.; Young, N. M.; Wakarchuk, W. W. *Nat. Biotechnol.* **1998**, *16*, 769-772.

162. Gilbert, M.; Brisson, J. R.; Karwaski, M. F.; Michniewicz, J.; Cunningham, A. M.; Wu, Y. Y.; Young, N. M.; Wakarchuk, W. W. *J. Biol. Chem.* **2000**, *275*, 3896-3906.

163. Ito, Y.; Paulson, J. C. *J. Am. Chem. Soc.* **1993**, *115*, 1603-1605.

164. Lubineau, A.; BassetCarpentier, K.; Auge, C. *Carbohydr. Res.* **1997**, *300*, 161-167.

165. Nilsson, K. G. I. *Carbohydr. Res.* **1989**, *188*, 9-17.

166. Palcic, M. M.; Venot, A. P.; Ratcliffe, R. M.; Hindsgaul, O. *Carbohydr. Res.* **1989**, *190*, 1-11.

167. Blixt, O.; Vasiliu, D.; Allin, K.; Jacobsen, N.; Warnock, D.; Razi, N.; Paulson, J. C.; Bernatchez, S.; Gilbert, M.; Wakarchuk, W. *Carbohydr. Res.* **2005**, *340*, 1963-1972.

168. Auge, C.; Gautheron, C. *Tetrahedron Lett.* **1988**, *29*, 789-790.

169. Hamamoto, T.; Takeda, S.; Noguchi, T. *Biosci. Biotech. Biochem.* **2005**, *69*, 1944-1950.

170. Liu, J. L. C.; Shen, G. J.; Ichikawa, Y.; Rutan, J. F.; Zapata, G.; Vann, W. F.; Wong, C. H. *J. Am. Chem. Soc.* **1992**, *114*, 3901-3910.

171. Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *J. Am. Chem. Soc.* **1988**, *110*, 7159-7163.

172. Yu, H.; Yu, H.; Karpel, R.; Chen, X. *Bioorg. Med. Chem.* **2004**, *12*, 6427-6435.

173. Ichikawa, Y.; Shen, G. J.; Wong, C. H. *J. Am. Chem. Soc.* **1991**, *113*, 4698-4700.

174. Ito, Y.; Paulson, J. C. *J. Am. Chem. Soc.* **1993**, *115*, 7862-7863.

175. Thiem, J.; Sauerbrei, B. *Angew. Chem. Int. Edit.* **1991**, *30*, 1503-1505.

176. Singh, S.; Scigelova, M.; Hallberg, M. L.; Howarth, O. W.; Schenkman, S.; Crout, D. H. G. *Chem. Com.* **2000**, 1013-1014.

177. Neubacher, B.; Schmidt, D.; Ziegelmuller, P.; Thiem, J. *Org. Biomol. Chem.* **2005**, *3*, 1551-1556.

178. Neubacher, B.; Svenja, S.; Kelm, S.; Frasch, A. C.; Meyer, B.; Thiem, J. *Chembiochem* **2006**, *7*, 896-899.

179. Vetere, A.; Paoletti, S. *FEBS Lett.* **1996**, *399*, 203-206.

180. Schmidt, D.; Sauerbrei, B.; Thiem, J. *J. Org. Chem.* **2000**, *65*, 8518-8526.

181. Perugino, G.; Cobucci-Ponzano, B.; Rossi, M.; Moracci, M. *Adv. Synth. Catal.* **2005**, *347*, 941-950.

182. Perugino, G.; Trincone, A.; Rossi, M.; Moracci, M. *Trends Biotechnol.* **2004**, *22*, 31-37.

183. Vaughan, M. D.; Johnson, K.; DeFrees, S.; Tang, X.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc.* **2006**, *128*, 6300-6301.

184. Bitton, R. J.; Guthmann, M. D.; Gabri, M. R.; Carnero, A. J. L.; Alonso, D. F.; Fainboim, L.; Gomez, D. E. *Oncol. Rep.* **2002**, *9*, 267-276.

185. Livingston, P. O.; Zhang, S. L.; Lloyd, K. O. *Cancer Immunol. Immunothr.* **1997**, *45*, 1-9.

186. Livingston, P. O.; Ragupathi, G. *Cancer Immunol. Immunothr.* **1997**, *45*, 10-19.

187. Karwaski, M. F.; Wakarchuk, W. W.; Gilbert, M. *Protein Expr. Purif.* **2002**, *25*, 237-240.

188. Gilbert, M.; Brisson, J. R.; Karwaski, M. F.; Michniewicz, J.; Cunningham, A. M.; Wu, Y. Y.; Young, N. M.; Wakarchuk, W. W. *J. Biol. Chem.* **2000**, *275*, 3896-3906.

189. Bernatchez, S.; Szymanski, C. M.; Ishiyama, N.; Li, J. J.; Jarrell, H. C.; Lau, P. C.; Berghuis, A. M.; Young, N. M.; Wakarchuk, W. W. *J. Biol. Chem.* **2005**, *280*, 4792-4802.

190. Malisan, F.; Testi, R. *B. B. A. -Mol. Cell. Biol. L.* **2002**, *1585*, 179-187.

191. Allende, M. L.; Proia, R. L. *Curr. Opin. Struct. Biol.* **2002**, *12*, 587-592.

192. Favaron, M.; Manev, H.; Alho, H.; Bertolino, M.; Ferret, B.; Guidotti, A.; Costa, E. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 7351-7355.

193. Malisan, F.; Testi, R. *Exp. Gerontol.* **2002**, *37*, 1273-1282.

194. Miljan, E. A.; Bremer, E. G. *Science's STKE* **2002**, 160.

195. de Paz, J. L.; Horlacher, T.; Seeberger, P. H. *Glycobiology* **2006**, *415*, 269-292.

196. Feizi, T.; Chai, W. G. *Nat. Rev. Mol. Cell. Bio.* **2004**, *5*, 582-588.

197. Feizi, T.; Fazio, F.; Chai, W. C.; Wong, C. H. *Curr. Opin. Struct. Biol.* **2003**, *13*, 637-645.

198. Timmer, M. S. M.; Stocker, B. L.; Seeberger, P. H. *Curr. Opin. Chem. Biol.* **2007**, *11*, 59-65.

199. De la Fuente, J. M.; Penades, S. *Biochim. Biophys. Acta* **2006**, *1760*, 636-651.

200. Koizumi, S. *Trends Glycosci. Glycotechnol.* **2003**, *15*, 65-74.

201. Antoine, T.; Bosso, C.; Heyraud, A.; Samain, E. *Biochimie* **2005**, *87*, 197-203.

202. Bettler, E.; Samain, E.; Chazalet, V.; Bosso, C.; Heyraud, A.; Joziasse, D. H.; Wakarchuk, W. W.; Imberty, A.; Geremia, R. A. *Glycoconj. J.* **1999**, *16*, 205-212.

203. Cottaz, S.; Samain, E. *Metab. Eng.* **2005**, *7*, 311-317.

204. Dumon, C.; Priem, B.; Martin, S. L.; Heyraud, A.; Bosso, C.; Samain, E. *Glycoconj. J.* **2001**, *18*, 465-474.

205. Priem, B.; Gilbert, M.; Wakarchuk, W. W.; Heyraud, A.; Samain, E. *Glycobiology* **2002**, *12*, 235-240.

206. Zhang, J. B.; Kowal, P.; Chen, X.; Wang, P. G. *Org. Biomol. Chem.* **2003**, *1*, 3048-3053.

207. Song, J. K.; Yao, Q. J.; Wang, P. G. *Glycobiology* **2006**, *16*, 1126-1126.

208. Song, J.; Zhang, H. C.; Li, L.; Bi, Z. S.; Chen, M.; Wang, W.; Yao, Q. J.; Guo, H. J.; Tian, M.; Li, H. F.; Yi, W.; Wang, P. G. *Curr. Org. Synth.* **2006**, *3*, 159-168.

209. Huang, G. L.; Mei, X. Y.; Zhang, H. C.; Wang, P. G. *J. Enzym. Inhib. Med. Chem.* **2006**, *21*, 597-599.

210. Shao, J.; Hayashi, T.; Wang, P. G. *Appl. Environ. Microbiol.* **2003**, *69*, 5238-5242.

211. Bettler, E.; Imberty, A.; Priem, B.; Chazalet, V.; Heyraud, A.; Joziasse, D. H.; Geremia, R. A. *Biochem. Biophys. Res. Commun.* **2003**, *302*, 620-624.

212. Mao, Z. C.; Shin, H. D.; Chen, R. R. Z. *Biotechnol. Prog.* **2006**, *22*, 369-374.

213. Samain, E. *Provisional Patent Application* **2007**.

214. Kovach, M. E.; Elzer, P. H.; Hill, D. S.; Robertson, G. T.; Farris, M. A.; Roop, R. M.; Peterson, K. M. *Gene* **1995**, *166*, 175-176.

215. Mengin-Lecreulx, D.; van Heijenoort, J. J. *J. Bacteriol* **1994**, *176*, 5788-5795.

216. Lee, S. Y. *Trends Biotechnol.* **1996**, *14*, 98-105.

217. Hakomori, S. I.; Igarashi, Y. *Adv. Lipid. Res.* **1993**, *25*, 147-162.

218. Saito, M. *Adv. Lipid. Res.* **1993**, *25*, 303-327.

219. Ledeen, R. W.; Wu, G. *Trends Glycosci. Glycotechnol.* **1992**, *4*, 174-187.

220. Zeng, G. C.; Gao, L. Y.; Birkle, S.; Yu, R. K. *Cancer Res.* **2000**, *60*, 6670-6676.

221. Birkle, S.; Zeng, G.; Gao, L.; Yu, R. K.; Aubry, J. *Biochimie* **2003**, *85*, 455-463.

222. Ruf, P.; Jager, M.; Ellwart, J.; Wosch, S.; Kusterer, E.; Lindhofer, H. *Int. J. Cancer* **2004**, *108*, 725-732.

223. Willison, H. J.; Townson, K.; Veitch, J.; Boffey, J.; Isaacs, N.; Andersen, S. M.; Zhang, P.; Ling, C. C.; Bundle, D. R. *Brain* **2004**, *127*, 680-691.

224. Karwaski, M. F.; Wakarchuk, W. W.; Gilbert, M. *Protein Expr. Purif.* **2002**, *25*, 237-240.

225. Cuatreca.P *Biochemistry (N.Y.)* **1973**, *12*, 3558-3566.

226. Holmgren, J.; Lindblad, M.; Fredman, P.; Svennerholm, L.; Myrvold, H. *Gastroenterology* **1985**, *89*, 27-35.

227. Townson, K.; Boffey, J.; Nicholl, D.; Veitch, J.; Bundle, D.; Zhang, P.; Samain, E.; Antoine, T.; Bernardi, A.; Arosio, D.; Sonnino, S.; Isaacs, N.; Willison, H. J. *Glycobiology* **2007**, *17*, 294-303.

228. Gilbert, M.; Brisson, J. R.; Karwaski, M. F.; Michniewicz, J.; Cunningham, A. M.; Wu, Y. Y.; Young, N. M.; Wakarchuk, W. W. *J. Biol. Chem.* **2000**, *275*, 3896-3906.

229. Hakomori, S. *Cancer Res.* **1985**, *45*, 2405-2414.

230. Livingston, P. O.; Wong, G. Y. C.; Adluri, S.; Tao, Y.; Padavan, M.; Parente, R.; Hanlon, C.; Calves, M. J.; Helling, F.; Ritter, G.; Oettgen, H. F.; Lloyd, J. O. *J. Clin. Oncol.* **1994**, *12*, 1036-1044.

231. Kennedy, R. C.; Shearer, M. H. *Int. Rev. Immunol.* **2003**, *22*, 141-172.

232. Vliegenthart, J. F. G. *FEBS Lett.* **2006**, *580*, 2945-2950.

233. Ada, G.; Isaacs, D. *Clin. Microbiol. Infec.* **2003**, *9*, 79-85.

234. Hermanson, G. T. In *Bioconjugate Techniques;* Academic Press: San Diego, 1996; pp 785.

235. Nitz, M.; Ling, C. C.; Otter, A.; Cutler, J. E.; Bundle, D. R. *J. Biol. Chem.* **2002**, *277*, 3440-3446.

236. Wu, X. Y.; Bundle, D. R. *J. Org. Chem.* **2005**, *70*, 7381-7388.

237. Bundle, D. R.; Rich, J. R.; Jacques, S.; Yu, H. N.; Nitz, M.; Ling, C. C. *Angew. Chem. Int. Edit.* **2005**, *44*, 7725-7729.

238. Wu, X. Y.; Lipinski, T.; Carrel, F. R.; Bailey, J. J.; Bundle, D. R. *Org. Biomol. Chem.* **2007**, *5*, 3477-3485.

239. Wu, X. Y.; Lipinski, T.; Paszkiewicz, E.; Bundle, D. R. *Chem. Eur. J.* **2008**, *14*, 6474-6482.

240. Ishioka, G. Y.; Lamont, A. G.; Thomson, D.; Bulbow, N.; Gaeta, F. C. A.; Sette, A.; Grey, H. M. *J. Immunol* **1992**, *148*, 2446-2451.

241. Leonard, E. G.; Canaday, D. H.; Harding, C. V.; Schreiber, J. R. *Infect. Immun.* **2003**, *71*, 4186-4189.

242. Nitz, M. Synthesis of *Trichinella spiralis* and *Candida albicans* Antigens containing β-Mannopyranosyl Epitopes, their Conformation and Immunochemistry, University of Alberta, 2001.

243. Mawas, F.; Niggemann, J.; Jones, C.; Corbel, M. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Infect. Immun.* **2002**, *70*, 5107-5114.

244. Buskas, T.; Li, Y. H.; Boons, G. J. *Chem. Eur. J.* **2004**, *10*, 3517-3524.

245. Wu, X.; Ling, C.; Bundle, D. R. *Org. Lett.* **2004**, *6*, 4407-4410.

246. Banchereau, J.; Briere, F.; Caux, C.; Davoust, J.; Lebecque, S.; Liu, Y. T.; Pulendran, B.; Palucka, K. *Annu. Rev. Immunol.* **2000**, *18*, 767-811.

247. Banchereau, J.; Steinman, R. M. *Nature* **1998**, *392*, 245-252.

248. Liu, Y. J. *Cell* **2001**, *106*, 259-262.

249. Guermonprez, P.; Valladeau, J.; Zitvogel, L.; Thery, C.; Amigorena, S. *Annu. Rev. Immunol.* **2002**, *20*, 621-667.

250. Lanzavecchia, A.; Sallusto, F. *Cell* **2001**, *106*, 263-266.

251. Banchereau, J.; Palucka, A. K. *Nat. Rev. Immunol.* **2005**, *5*, 296-306.

252. Steinman, R. M.; Banchereau, J. *Nature* **2007**, *449*, 419-426.

253. Fernandez, N. C.; Lozier, A.; Flament, C.; Ricciardi-Castagnoli, P.; Bellet, D.; Suter, M.; Perricaudet, M.; Tursz, T.; Maraskovsky, E.; Zitvogel, L. *Nat. Med.* **1999**, *5*, 405-411.

254. Lucas, M.; Schachterle, W.; Oberle, K.; Aichele, P.; Diefenbach, A. *Immunity* **2007**, *26*, 503-517.

255. Palucka, A. K.; Ueno, H.; Connolly, J.; Kerneis-Norvell, F.; Blanck, J. P.; Johnston, D. A.; Fay, J.; Banchereau, J. *J. Immunother.* **2006**, *29*, 545-557.

256. O'Rourke, M. G. E.; Johnson, M.; Lanagan, C.; See, J.; Yang, J.; Bell, J. R.; Slater, G. J.; Kerr, B. M.; Crowe, B.; Purdie, D. M.; Elliott, S. L.; Ellem, K. A. O.; Schmidt, C. W. *Cancer Immunol. Immunother.* **2003**, *52*, 387-395.

257. Dudziak, D.; Kamphorst, A. O.; Heidkamp, G. F.; Buchholz, V. R.; Trumpfheller, C.; Yamazaki, S.; Cheong, C.; Liu, K.; Lee, H. W.; Park, C. G.; Steinman, R. M.; Nussenzweig, M. C. *Science* **2007**, *315*, 107-111.

258. Delneste, Y.; Magistrelli, G.; Gauchat, J. F.; Haeuw, J. F.; Aubry, J. P.; Nakamura, K.; Kawakami-Honda, N.; Goetsch, L.; Sawamura, T.; Bonnefoy, J. Y.; Jeannin, P. *Immunity* **2002**, *17*, 353-362.

259. Jiang, W. P.; Swiggard, W. J.; Heufler, C.; Peng, M.; Mirza, A.; Steinman, R. M.; Nussenzweig, M. C. *Nature* **1995**, *375*, 151-155.

260. Witmerpack, M. D.; Swiggard, W. J.; Mirza, A.; Inaba, K.; Steinman, R. M. *Cell. Immunol.* **1995**, *163*, 157-162.

261. Inaba, K.; Swiggard, W. J.; Inaba, M.; Meltzer, J.; Mirza, A.; Sasagawa, T.; Nussenzweig, M. C.; Steinman, R. M. *Cell. Immunol.* **1995**, *163*, 148-156.

262. Mahnke, K.; Guo, M.; Lee, S.; Sepulveda, H.; Swain, S. L.; Nussenzweig, M.; Steinman, R. M. *J. Cell Biol.* **2000**, *151*, 673-683.

263. Bonifaz, L.; Bonnyay, D.; Mahnke, K.; Rivera, M.; Nussenzweig, M. C.; Steinman, R. M. *J. Exp. Med.* **2002**, *196*, 1627-1638.

264. Steinman, R. M.; Pope, M. *J. Clin. Invest.* **2002**, *109*, 1519-1526.

265. Trumpfheller, C.; Finke, J. S.; Lopez, C. B.; Moran, T. M.; Moltedo, B.; Soares, H.; Huang, Y. X.; Schlesinger, S. J.; Park, C. G.; Nussenzweig, M. C.; Granelli-Piperno, A.; Steinman, R. M. *J. Exp. Med.* **2006**, *203*, 607-617.

266. Soares, H.; Waechter, H.; Glaichenhaus, N.; Mougneau, E.; Yagita, H.; Mizenina, O.; Dudziak, D.; Nussenzweig, M. C.; Steinman, R. M. *J. Exp. Med.* **2007**, *204*, 1095-1106.

267. Soares, H.; Waechter, H.; Glaichenhaus, N.; Mougneau, E.; Yagita, H.; Mizenina, O.; Dudziak, D.; Nussenzweig, M. C.; Steinman, R. M. *J. Exp. Med.* **2007**, *204*, 1095-1106.

268. van Broekhoven, C. L.; Parish, C. R.; Demangel, C.; Britton, W. J.; Altin, J. G. *Cancer Res.* **2004**, *64*, 4357-4365.

269. Bonifaz, L. C.; Bonnyay, D. P.; Charalambous, A.; Darguste, D. I.; Fujii, S. I.; Soares, H.; Brimnes, M. K.; Moltedo, B.; Moran, T. M.; Steinman, R. M. *J. Exp. Med.* **2004**, *199*, 815-824.

270. Hawiger, D.; Inaba, K.; Dorsett, Y.; Guo, M.; Mahnke, K.; Rivera, M.; Ravetch, J. V.; Steinman, R. M.; Nussenzweig, M. C. *J. Exp. Med.* **2001**, *194*, 769-779.

271. Wang, W. W.; Das, D.; Tang, X. L.; Budzynski, W.; Suresh, M. R. *J. Immunol. Methods* **2005**, *306*, 80-92.

272. Cao, Y.; Christian, S.; Suresh, M. R. *J. Immunol. Methods* **1998**, *220*, 85-91.

273. Cao, Y.; Suresh, M. R. *Bioconjug. Chem.* **1998**, *9*, 635-644.

274. Herzenberg, L. A.; Tokuhisa, T.; Herzenberg, L. A. *Nature* **1980**, *285*, 664-667.

275. Peeters, C. C. A. M.; Tenbergenmeekes, A. M.; Poolman, J. T.; Beurret, M.; Zegers, B. J. M.; Rijkers, G. T. *Infect. Immun.* **1991**, *59*, 3504-3510.

276. Herzenberg, L. A.; Tokuhisa, T. *J. Exp. Med.* **1982**, *155*, 1730-1740.

277. Schutze, M. P.; Leclerc, C.; Jolivet, M.; Audibert, F.; Chedid, L. *J. Immunol.* **1985**, *135*, 2319-2322.

278. Jacob, C. O.; Arnon, R.; Sela, M. *Mol. Immunol.* **1985**, *22*, 1333-1339.

279. Davis, B. G. *Chem. Rev.* **2002**, *102*, 579-601.

280. Konenwaisman, S.; Fridkin, M.; Cohen, I. R. *J. Immunol.* **1995**, *154*, 5977-5985.

281. Amir-Kroll, H.; Riveron, L.; Sarmiento, M. E.; Sierra, G.; Acosta, A.; Cohen, I. R. *Vaccine* **2006**, *24*, 6555-6563.

282. Amir-Kroll, H.; Nussbaum, G.; Cohen, I. R. *J. Immunol.* **2003**, *170*, 6165-6171.

283. Carpino, L. A.; Han, G. Y. *J. Am. Chem. Soc.* **1970**, *92*, 5748.

284. Dourtoglou, V.; Gross, B.; Lambropoulou, V.; Zioudrou, C. *Synthesis-Stuttgart* **1984**, 572-574.

285. Chhabra, S. R.; Hothi, B.; Evans, D. J.; White, P. D.; Bycroft, B. W.; Chan, W. C. *Tetrahedron Lett.* **1998**, *39*, 1603-1606.

286. Wittmann, V.; Seeberger, S. *Angew. Chem. Int. Edit.* **2000**, *39*, 4348.

287. Wang, S. S. *J. Am. Chem. Soc.* **1973**, *95*, 1328-1333.

288. Palcic, M. M.; Heerze, L. D.; Pierce, M.; Hindsgaul, O. *Glycoconj. J.* **1988**, *5*, 49.

289. Ohlsson, J.; Magnusson, G. *Tetrahedron* **2000**, *56*, 9975-9984.

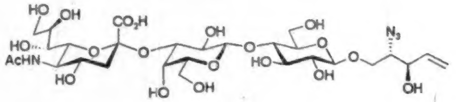
290. Gao, Y.; Eguchi, A.; Kakehi, K.; Lee, Y. C. *Org. Lett.* **2004**, *6*, 3457.

Appendix 1

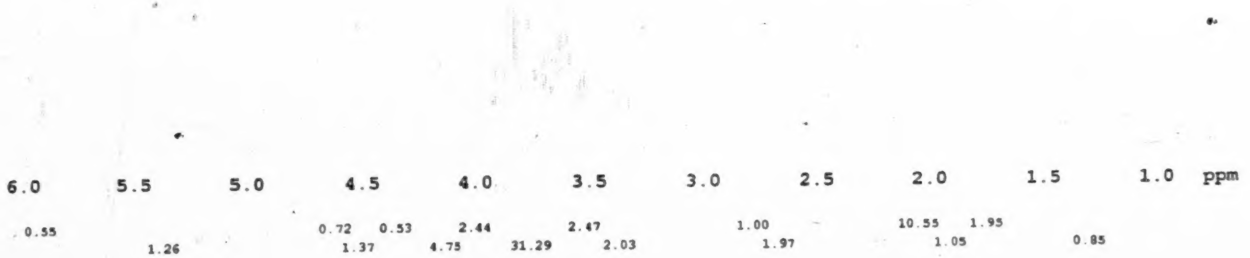
NMR Spectra of Gangliosides Produced by the Living Factory Approach

600 MHz 1D in D20 (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 28.0 C -> actual temp = 27.0 C, id600 probe

Pulse Sequence: s2pul

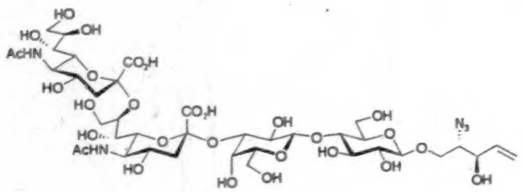

HO
OH
HO
AcHN
HO
CO_2H
O
OH
O
HO
OH
O
OH
HO
O
OH
O
N_3
OH

GM$_3$ analogue **24**

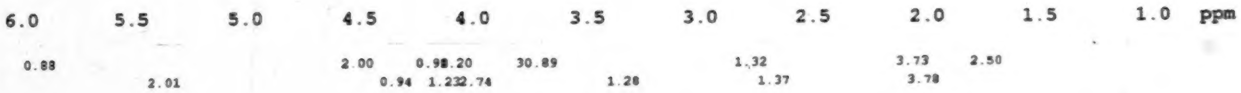


600 MHz 1D in D2O (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 28.0 C -> actual temp = 27.0 C, id600 probe

Pulse Sequence: s2pul

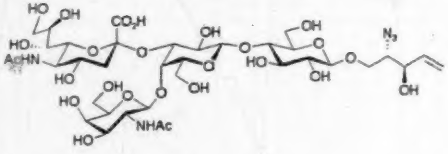


GD$_3$ analogue **25**

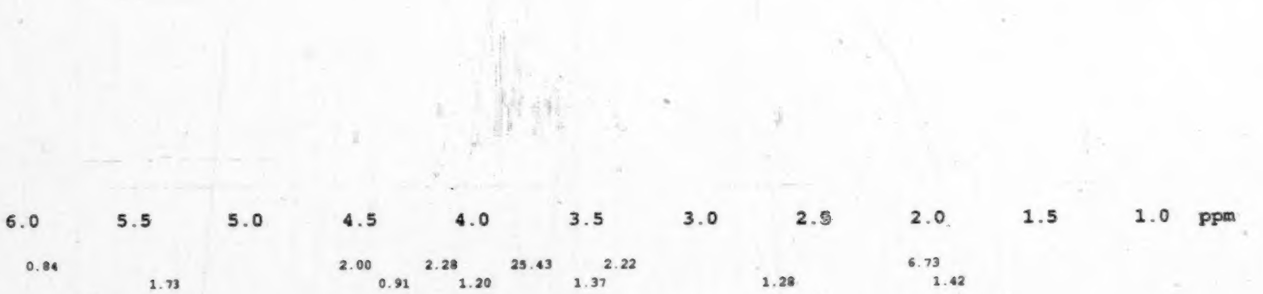


600 MHz 1D in D2O (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 28.0 C -> actual temp = 27.0 C, id600 probe

Pulse Sequence: s2pul

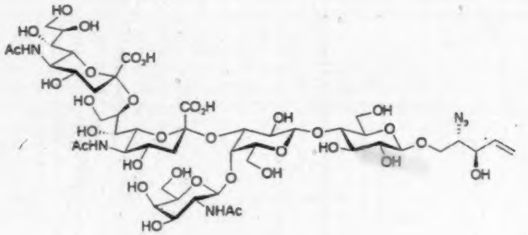

HO
OH
HO
AcHN
CO2H
O
HO
OH
HO
NHAc
HO
OH
OH
O
HO
OH
OH
N3
OH

GM$_2$ analogue **26**

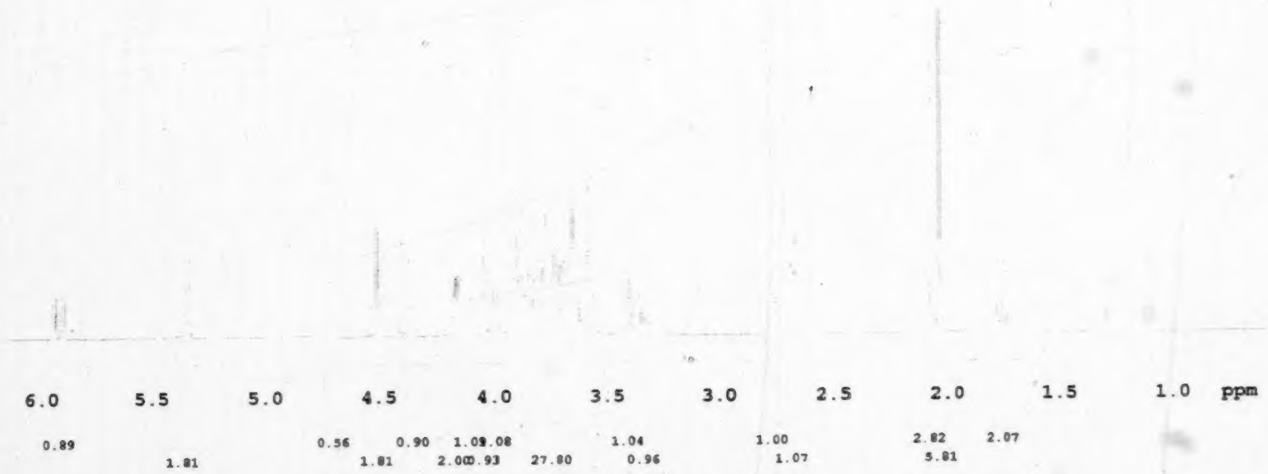


600 MHz 1D in D2O (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 28.0 C -> actual temp = 27.0 C, id600 probe

Pulse Sequence: s2pul

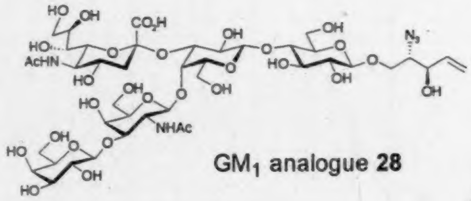

HO
OH
HO
AcHN
CO₂H
HO
HO
O
CO₂H
HO
AcHN
O
OH
O
OH
HO
OH
O
HO
OH
OH
NHAc
HO
N₃
OH

GD_2 analogue **27**

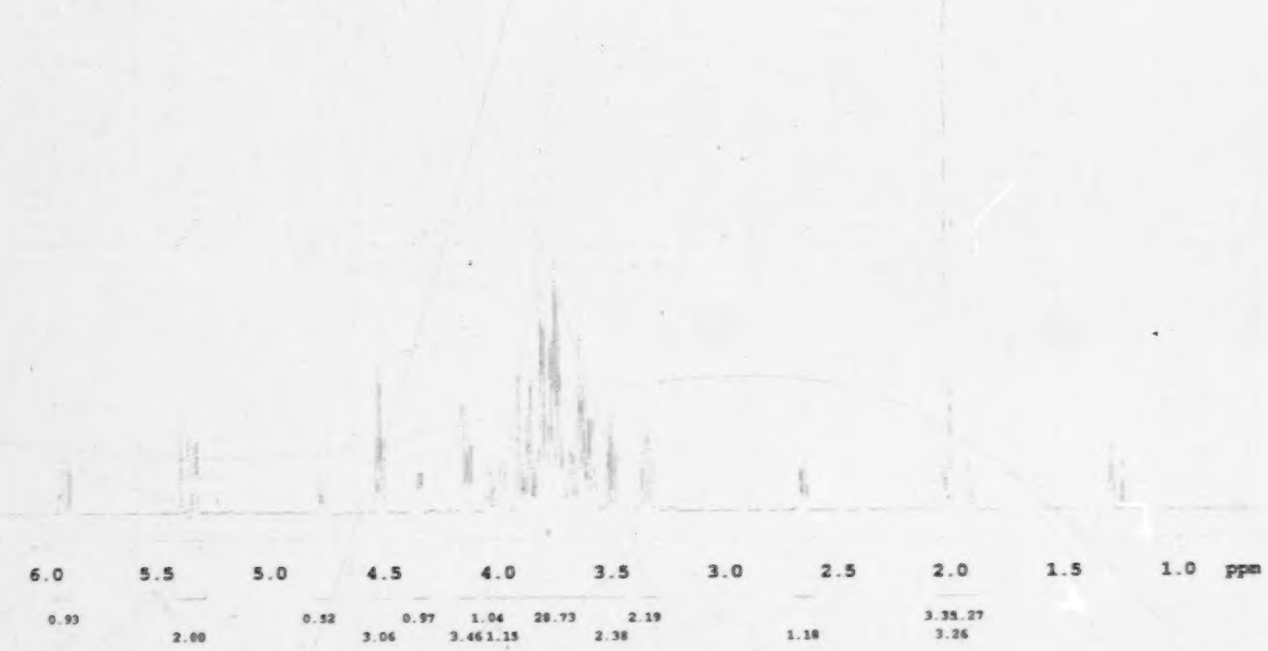


600 MHz 1D in D2O (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 28.0 C -> actual temp = 27.0 C, id600 probe

Pulse Sequence: s2pul

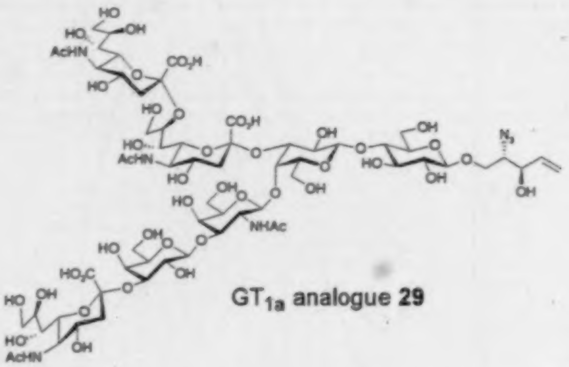

HO
OH
CO₂H
HO
AcHN
HO
O
OH
OH
O
HO
OH
OH
N₃
OH
HO
OH
NHAc
HO
OH
OH
HO

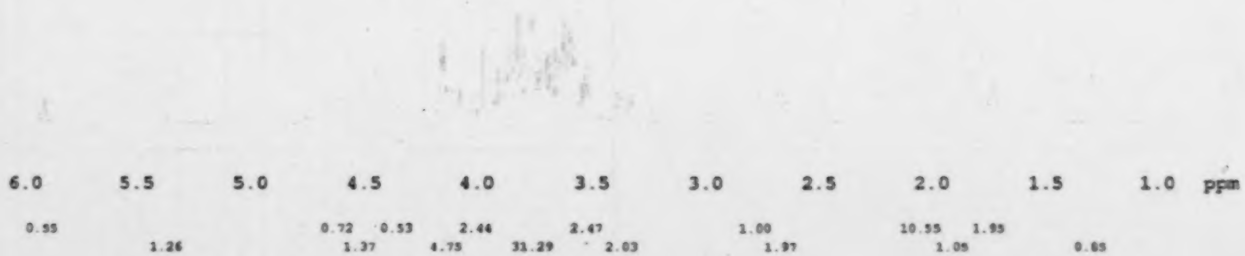
GM$_1$ analogue **28**



600 MHz 1D in D2O (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 28.0 C -> actual temp = 27.0 C, id600 probe

Pulse Sequence: s2pul


HO
HO
OH
AcHN
CO2H
HO
HO
CO2H
HO
AcHN
HO
OH
OH
OH
O
HO
OH
OH
N3
OH
HO
OH
NHAc
HO
OH
OH
HO2C
HO
OH
HO
AcHN
OH

GT_{1a} analogue **29**